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AN EVALUATION OF IN VITRO TESTS IN THE DIAGNOSIS OF VIRUS DISEASES

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Dallas, Texas

THE neutralization test is the laboratory procedure now in general use for the diagnosis of virus diseases. Since this test is cumbersome and costly, as well as time-consuming, efforts have been made in recent years to replace it with methods which would be simple, accurate, and inexpensive. The complement fixation test recently described by Casals and Palacios[1] and by Havens and his associates[2] is sufficiently specific to differentiate the various experimental infections caused by the neurotropic viruses. This test has already yielded encouraging results[3, 4] when used in the diagnosis of human infections induced by some of these viruses. Meanwhile, certain other in vitro antigen-antibody tests have been described by Goodner,[5] Cannon and Marshall,[6] and Roberts and Jones,[7-9] but no attempts to determine the value of these tests as diagnostic aids seem to have been published to date. It appeared that comparison of the efficiency of these tests with that of the aforementioned complement fixation and neutralization tests might lead to further simplification of laboratory methods. In the present study, therefore, attempts have been made to ascertain the relative value and practicability of these procedures in the diagnosis of virus diseases. In a few experiments with the influenza virus, the Hirst[10] red cell agglutination-inhibition test was also included in this comparison.

MATERIALS AND METHODS

All antigens were freshly prepared and the technique as outlined by the authors of the respective tests was rigidly adhered to in all details. Antigens used in this study were prepared with the viruses of eastern and western equine encephalomyelitis,[1, 5-7] St. Louis encephalitis,[1, 5-7] rabies,[1, 5-7] lymphogranuloma venereum,[5-7, 11] and type A influenza.[5-7, 12] Human as well as hyperimmune animal sera† were tested. In a few instances hyperimmune mouse serum was obtained by injecting animals with homologous brain tissue and pooling the samples of blood. In the complement fixation tests with the neurotropic viruses, all human, goat, and mouse sera were heated to 60° C. for twenty minutes before using. Guinea pig and rabbit sera, respectively, were inactivated for twenty minutes at 56° C. In the complement fixation tests with the viruses of epidemic influenza and lymphogranuloma venereum the sera were inactivated for thirty minutes at 56° C.

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*Now serving in the Sanitary Corps, United States Army.

†The authors wish to express their appreciation to the following persons for material used in this study: Drs. S. C. Bukantz, H. R. Cox, C. G. Harford, Marion Howard, B. F. Howitt, Anson Hoyt, Joseph Tamura, L. R. Vawter. The sera from the human cases of equine encephalomyelitis (western type), which occurred during the 1941 outbreak in the central northwest, were supplied by Dr. C. M. Eklund.

TABLE I

RESULTS OF AGGLUTINATION, COMPLEMENT FIXATION, AND NEUTRALIZATION TESTS ON REPRESENTATIVE SERUMS

ANTIGENS		SERA*	TEST				
VIRUS	INFECTED TISSUE		COLLODION-FIXATION (GOODNER[5])	COLLODION-AGGLUTINATION (CANNON-MARSHALL[6])	B. A. METHOD (ROBERTS-JONES[7])	COMPLEMENT FIXATION[1, 11, 12]	NEUTRALIZATION[13-15]
St. Louis encephalitis (Taylor strain)	Mouse brain	Rabbit	⊕	+	+	ac	+
		Horse†‡	⊕	⊕	⊕	ac	+
		Mouse	○	⊕	○	+	+
		Human†	⊕	+	⊕	⊕	⊕
Western equine encephalomyelitis (California strain)	Mouse brain	Guinea pig	○	○	○	+	+
		Rabbit	ns	ns	ns	+	+
		Horse†	⊕	+	+	+	+
		Human†	ns	ns	ns	ac	+
		Human†	⊕	⊕	⊕	+	+
		Human§	○	○	○	○	⊕
Eastern equine encephalomyelitis (TenBroeck strain)	Mouse brain	Guinea pig	⊕	+	+	+	+
		Rabbit	○	○	○	○	⊕
		Rabbit	⊕	⊕	⊕	ac	+
		Guinea pig	⊕	+	⊕	⊕	⊕
Rabies (No. 3557)	Mouse brain	Guinea pig	⊕	+	⊕	+	⊕
		Rabbit	+	⊕	+	ac	+
		Goat	⊕	+	⊕	⊕	⊕
		Human‖	⊕	+	+	+	+
		Human†¶	○	⊕	○	○	○
Influenza (PR-8)	Mouse lung	Human†	+	+	⊕	+	+
		Human†	+	⊕	⊕	+	+
		Human**	⊕	+	⊕	○	+
		Human**	+	⊕	⊕	+	+
	Allantoic fluid	Ferret	+	+	+	+	+
		Human†	+	+	+	+	+
		Human†	+	⊕	+	+	+
Lymphogranuloma venereum (J)	Yolk sac	Goat	ns	ns	ns	ac	nt
		Human†	+	+	⊕	⊕	nt
		Human†	+	+	+	+	nt
		Human†	+	+	⊕	⊕	nt

*Unless otherwise stated, the sera were obtained by hyperimmunizing animals.

†Natural infection; convalescent phase serum sample.

‡Tested previously by Hammon and Izumi and found to contain neutralizing antibodies against the virus of St. Louis encephalitis.

§Person vaccinated against western equine virus.

‖Serum was obtained twenty days after exposure and eleven days after completion of Pasteur treatment.

¶Patient with clinical rabies; no Pasteur treatment; serum obtained thirty-nine days after exposure.

**Vaccinated with influenza a complex vaccine.[16]

\+ Indicates a positive test.

⊕ Indicates a doubtful test; weakly positive neutralization test.

○ Indicates a negative reaction.

ac, Serum anticomplementary.

ns, Nonspecific reaction; namely, reaction with test antigen and with control.

nt, Not tested.

The neutralization tests with the viruses of St. Louis encephalitis and equine encephalomyelitis were performed according to the method previously described by Hammon and Izumi,[13] while with the rabies virus the procedure of Webster[14] was used. The technique of the neutralization test with the influenza

virus was essentially the same as that described by Lennette and Horsfall.[15] No neutralization tests were conducted with the agent of lymphogranuloma venereum. In reading the agglutination tests, the method of resuspension-agglutination was used since this procedure tends to eliminate prozone effects. The suspension of collodion particles, together with the antigen-antibody system in question, is centrifuged, thus bringing the particles together.[6]

RESULTS

Some of the tests herein reported were conducted during the latter part of 1941 and involved the use of sera which had been on hand since 1937. The results of the tests on representative serum specimens from various natural and induced virus infections and from immunized animals are summarized in Table I. Few of the sera tested gave positive results with all of the tests used. In the majority of instances there was agreement between the results of the complement fixation and neutralization tests, while in only a few instances did the results of agglutination tests agree with those obtained by the other procedures. The serum from one of the workers (E. M. I.) who had been previously vaccinated against the western equine virus contained neutralizing antibodies, but no complement-fixing or agglutinating antibodies could be demonstrated. A number of sera from individuals inoculated[16] with the complex influenza a-distemper chick embryo vaccine of Horsfall and Lennette[17] were also tested by the various methods, using infected mouse lung tissue in preparing the antigens. The results with only two of these sera are presented in Table I. Although no complement-fixing antibodies could be demonstrated in one of these serum samples, both neutralizing and agglutinating antibodies were detectable. Aside from the serum which failed to show complement-fixing antibodies, a remarkable degree of correlation was noted with influenza virus and the corresponding serum. Incidentally, all of these gave positive reactions with the Hirst red cell agglutination-inhibition technique.[10]

With the rabies and influenza viruses suggestive flocculation occurred with some sera even without the use of collodion particles or bacterial cells. These observations are in agreement with those previously reported by Havens and Mayfield[18, 19] and by Magill and Francis.[20] Henle and Chambers[21] also observed flocculation when the pooled extraembryonic fluids of developing chick embryos inoculated with influenza a virus were mixed with human or ferret convalescent sera.

The frequent lack of agreement between the complement fixation and neutralization tests has been demonstrated previously[4] and may be influenced by the time during convalescence when the blood specimens are obtained. The temperature at which the serum is stored may likewise influence the results.[2] Neutralizing antibodies against the western equine encephalomyelitis virus usually may be detected earlier than the complement-fixing antibodies, while in St. Louis encephalitis the reverse is true.[4, 22, 23] Furthermore, the complement-fixing antibodies persist longer in equine encephalomyelitis than they do in St. Louis encephalitis.[23]

Many factors appear to complicate the procedure involving the use of collodion particles or bacterial cells. Apparently particles 24 hours old or older

often flocculate spontaneously in the presence of serum proteins (especially whole horse serum); hence low serum dilutions may yield false positive results. Also, fresh human serum contains factors which may cause spontaneous flocculation. This, however, may be eliminated by inactivating the serum at 56° C. for thirty minutes. Similar difficulties present themselves also when bacteria instead of collodion particles are used (Roberts-Jones test). Spontaneous clumping frequently occurs in the freshly prepared suspensions of bacterial cells killed by flowing steam. This was demonstrated by examination of stained preparations.

SUMMARY AND CONCLUSIONS

An analysis of these findings indicates that the Goodner collodion-fixation test, the Cannon and Marshall collodion-agglutination test, and the Roberts and Jones bacterial agglutination method, though simple in operation, present too many complicating factors to permit of general use as routine procedures. Because of the ease with which many sera can be examined and because of the relative simplicity of the technique, the complement fixation test,* whether used alone or in conjunction with the neutralization test, is eminently useful as a method for diagnosis of virus diseases.

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*At the present time antigens are being prepared and tested for their usefulness with the view of recommending their preparation on a commercial scale for distribution to laboratories.

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THE CEPHALIN-CHOLESTEROL FLOCCULATION TEST

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AN INTEREST in the treatment of cirrhosis of the liver has led quite naturally to a consideration of the value of liver function tests as an aid to the early diagnosis of cirrhosis and as a measure of change in hepatic function produced by therapy. The known limitations of the older tests prompted us to investigate the suitability of the cephalin-cholesterol flocculation test.

The cephalin-cholesterol flocculation test was introduced in 1938 by Hanger.[3] He described the preparation of the cephalin-cholesterol emulsion as well as the results obtained in a variety of diseases. No significant flocculation was observed in the absence of liver disease, and variable degrees of flocculation depending upon the severity of the lesion) were consistently found in the presence of hepatic disease. Hanger and others,[1, 4-13] have confirmed these findings and have established beyond any doubt the usefulness of the test. Differences of opinion have arisen, however, concerning the value of the test in the presence of other systemic disease, its correlation with other liver function tests, its usefulness in differentiating obstructive from nonobstructive jaundice, the mechanism of a positive reaction, and other problems. The possibility that the present data may contribute to a better understanding of some of these problems would seem to justify this communication.

MATERIAL

From among the 1,500 tests reviewed, 500 were chosen for this analysis. They were done on the sera of patients whose diagnoses have been established in most instances at necropsy or at operation. The remaining diagnoses are supported by unmistakable clinical evidence. The cases have been classified in three groups:

I. Patients with known lesions of the liver
   a. Diffuse
   b. Discrete

II. Patients with suspected or probable dysfunction of the liver

III. Patients with no known lesion or dysfunction of the liver

METHOD

Inasmuch as not more than from ten to fifteen tests were done per week, it was found advantageous to do them only one morning each week. This simplified the laboratory work and prevented needless waste of reagents.

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From the Department of Medicine and the Snodgrass Laboratories of the St. Louis City Hospital, and the Department of Medicine, Washington University School of Medicine.

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The procedure was as follows:

A stock ether solution was prepared by placing 8 c.c. of ether in the vial containing the cephalin-cholesterol mixture* and shaking thoroughly. The resultant stock ether solution was stored in the refrigerator for use as needed.

Not more than a few hours prior to the actual testing of sera an emulsion was prepared by adding (with constant stirring) 1 c.c. of the stock ether solution to 35 c.c. of freshly distilled water which had been warmed to 65 or 70° C. This mixture was then heated slowly to boiling and allowed to simmer until the final volume reached 30 c.c. After cooling to room temperature, the emulsion was ready for use. That which was not used within a few hours was discarded.

One cubic centimeter of this emulsion was placed in a centrifuge tube containing 0.2 c.c. of the patient's serum diluted with 4 c.c. of physiologic saline (0.85 per cent sodium chloride solution). The mixture was shaken thoroughly, stoppered with cotton, and permitted to stand undisturbed at room temperature for twenty-four hours. The reactions were graded in terms of negative and 1 to 4 plus, a 4 plus indicating complete flocculation which left the supernatant fluid water-clear. A control tube containing 4 c.c. of saline and 1 c.c. of emulsion without serum was always made to test the stability of the emulsion.

It is essential that all glassware be scrupulously clean. If sera are permitted to stand for six hours or longer, false positive reactions will occur with normal sera and increasingly positive reactions with abnormal sera. Knowing that this invariably occurs, we have dispensed with forty-eight-hour readings.

RESULTS

The sera of 178 patients with known diffuse parenchymatous liver disease brought about flocculation of the cephalin-cholesterol emulsion in 173 instances (Table I). Only five (or 2.8 per cent) were read as negative reactions. Two of these occurred in the presence of cirrhosis which anatomically was early but nonetheless definite. Another occurred in the presence of carcinoma of the liver and a fourth in the presence of diffuse atrophy of the liver. The fifth negative test was obtained in a late case of catarrhal jaundice; it is felt that the test would undoubtedly have been positive at an earlier time.

TABLE I

KNOWN LESIONS OF THE LIVER—DIFFUSE

NUMBER OF CASES	DIAGNOSIS	NEGATIVE 0	NEGATIVE 1 PLUS	POSITIVE 2 PLUS	POSITIVE 3 PLUS	POSITIVE 4 PLUS
127	Cirrhosis	2	0	4	48	73
32	Catarrhal jaundice	1	0	3	7	21
2	Carcinoma of liver	0	1	0	0	1
8	Acute hepatitis	0	0	0	2	6
5	Atrophy of liver	0	1	0	2	2
2	Thrombosis of the portal vein	0	0	0	0	2
1	Hand-Christian-Schüller disease	0	0	0	0	1
1	CCl_4 poisoning	0	0	0	1	0
Total 178		3	2	7	60	106
100 per cent		1.7	1.1	3.9	33.7	59.6

Negative, 2.8 per cent
Positive, 97.2 per cent

Discrete lesions of the liver, involving only a small part of the parenchyma, were associated with a significant flocculation in 53.3 per cent of the instances (Table II). Attention was usually, but not always, directed to these lesions by

*We wish to express our thanks to Dr. David Klein, of the Wilson Laboratories, who so generously furnished these mixtures to us.

the presence of obstructive jaundice. There was, however, no correlation between the presence, duration, or severity of the jaundice in these cases and the presence or absence of flocculation. The positive reaction in the patient with liver abscess may or may not be significant, for a pyogenic process anywhere in the body, as will be emphasized later, may produce a positive test.

TABLE II

KNOWN LESIONS OF THE LIVER—DISCRETE

NUMBER OF CASES	DIAGNOSIS	NEGATIVE		POSITIVE		
		0	1 PLUS	2 PLUS	3 PLUS	4 PLUS
40	Carcinoma (metastatic or direct extension into liver or bile ducts)	17	3	9	5	6
3	Cysts of liver	0	0	0	3	0
1	Abscess of liver	0	0	1	0	0
1	Bullet wound of liver	1	0	0	0	0
Total 45		18	3	10	8	6
100 per cent		40.0	6.7	22.2	17.8	13.3
	Negative, 46.7 per cent Positive, 53.3 per cent					

In Table III are grouped those cases in which a reasonable suspicion of dysfunction of the liver existed, even though in some instances there may have been no demonstrable anatomical lesion of the liver. Of these 180 cases, sixty-seven were associated with definite flocculation (37.2 per cent). Cholecystitis and cholelithiasis were responsible for twenty-one of these, chronic passive congestion for twenty-four, and chronic alcoholism for eight. No correlation could be established between the duration of cholecystitis and cholelithiasis and the presence or absence of flocculation. Negative reactions were encountered in patients whose history extended over a fifteen- to twenty-year period and positive reactions in patients with histories of only one to three months' duration. The diagnosis of cardiac cirrhosis is not warranted on the basis of a positive flocculation, for autopsy findings have not been confirmatory. The possibility of detecting early cirrhosis among patients suffering from alcoholism has been studied carefully. Although 20 per cent of such patients had a significant flocculation, none has returned with clinical cirrhosis even though two to three years have lapsed in some instances.

TABLE III

SUSPECTED OR PROBABLE DYSFUNCTION OF THE LIVER

NUMBER OF CASES	DIAGNOSIS	NEGATIVE		POSITIVE		
		0	1 PLUS	2 PLUS	3 PLUS	4 PLUS
44	Cholecystitis and cholelithiasis	20	3	6	8	7
40	Chronic alcoholism with varying degrees of icterus and hepatomegaly	27	5	1	4	3
55	Cardiac disease with chronic passive congestion	24	7	8	8	8
4	Congenital hemolytic icterus	2	0	0	0	2
16	Eclampsia	5	5	1	3	2
18	Diabetes mellitus	12	0	2	1	3
3	Hyperthyroidism	3	0	0	0	0
Total 180		93	20	18	24	25
100 per cent		51.7	11.1	10.0	13.3	13.9
	Negative, 62.8 per cent Positive, 37.2 per cent					

A control series of 105 tests is summarized in Table IV. Those from subjects without known disease (interns, residents, and laboratory technicians) were negative in all instances. Flocculations thought to be significant did occur, however, in nineteen patients who were not suspected of having any liver disease. Twelve of the nineteen positive reactions occurred in the presence of infection. This has been noted repeatedly, but unfortunately not all instances are sufficiently clear cut to permit inclusion in this paper. It has become the custom in this laboratory to disregard positive reactions in the presence of infectious disease except as a possible indication of transient dysfunction to be expected with other systemic diseases. If one corrects the percentage of positive reactions accordingly, there remain 3.9 per cent of unpredictable and unaccountable "false positives."

TABLE IV

NO KNOWN LESION OR DYSFUNCTION OF THE LIVER

NUMBER OF CASES	DIAGNOSIS	NEGATIVE	POSITIVE			
		0	1 PLUS	2 PLUS	3 PLUS	4 PLUS
61	Normal (interns, etc.)	59	2	0	0	0
3	Pernicious anemia	3	0	0	0	0
6	Tuberculosis	4	0	0	2*	0
1	Mesenteric artery thrombosis	0	0	0	1	0
4	Pancreatitis	3	0	0	1	0
2	Leucemia	2	0	0	0	0
3	Malaria	1	0	0	1	1
1	Constrictive pericarditis	1	0	0	0	0
1	Boeck's sarcoid	0	0	1	0	0
1	Pyelonephritis	0	0	0	0	1
6	Glomerulonephritis	4	1	0	1	0
1	Renal dwarfism	0	0	0	1	0
6	Lobar pneumonia	3	0	0	2	1
9	Abscess formation	3	0	3	1	2
Total 105		83	3	4	10	5
100 per cent		79.0	2.9	3.8	9.5	4.8
	Negative, 81.9 per cent Positive, 18.1 per cent					

*Complicated by empyema or lung abscess.

It is desirable to check further the accuracy of the test by comparison with other hepatic function tests. In Table V is indicated the percentage of positive tests obtained with the cephalin-cholesterol flocculation test, the bromsulfalein test,* the intravenous hippuric acid test,† and the serum diastase test.‡

TABLE V

COMPARISON OF CEPHALIN-CHOLESTEROL FLOCCULATION TEST WITH OTHER LIVER FUNCTION TESTS

NUMBER OF CASES*	DIAGNOSIS	PER CENT POSITIVE TESTS			
		C.C.	I.V.HIPP.	B.S.P.	DIASTASE
127	Cirrhosis	98.5	83.1	89.1	62.3
32	Catarrhal jaundice	96.8	58.3	50.0	56.6
44	Cholecystitis and cholelithiasis	47.7	66.7	---	44.8
40	Chronic alcoholism	20.0	35.7	66.7	34.3
40	Carcinoma of liver or biliary tract	50.0	100.0	100.0	65.3

*All four tests were done in at least 75 per cent of the cases.

*Mateer's modification of the Rosenthal test was used[8]. A single blood specimen was removed forty-five minutes following the injection of 5 mg. of the dye per kilogram of body weight. Any retention was considered abnormal.

†A urine specimen was collected exactly one hour following the injection of 1.77 Gm. of sodium benzoate (equivalent to 1.5 Gm. of benzoic acid).[9, 17] Excretion of less than 1.0 Gm. of hippuric acid (equivalent to 0.80 Gm. of sodium benzoate) was considered abnormal.

‡A diastase level of 60 Somogyi units or less was considered abnormal.[2]

With diffuse parenchymatous liver disease, the cephalin-cholesterol test surpassed the other tests used in the frequency with which supposed hepatic dysfunction was detected. On the other hand, the intravenous hippuric acid and the bromsulfalein tests both gave more frequent indication of liver disease in the presence of discrete or focal lesions of the liver.

A decrease in serum albumin with a compensatory increase in globulin is frequently associated with liver disease.[16] Obviously, loss of albumin as in renal disease or increase in globulin with chronic infection will result in similar serum protein patterns. An effort has been made therefore to collect instances of all sorts with disturbances of albumin-globulin ratios. Almost all cases of cirrhosis had albumin-globulin ratios of less than 1:4. Since these cases form such a large part of this series, it is not surprising that 89.5 per cent of the 124 with low ratios had positive flocculations. It is striking, however, that 10.5 per cent of these had negative cephalin-cholesterol flocculation tests, indicating that relative increases in the globulin fraction are not necessarily associated with a positive flocculation. Conversely, 45.4 per cent of those with "normal" ratios had positive flocculations indicating that increase in the globulin fraction need not occur or may be very slight in the presence of a positive test.

An interesting observation, not included in Tables I to V, has to do with the flocculation test in pregnancy and in the newborn infant (Table VI). In studying jaundice of the newborn infant, it was noted that 66.7 per cent of fifteen such infants had positive flocculation tests. The sera of eleven nonjaundiced

TABLE VI

CEPHALIN-CHOLESTEROL FLOCCULATION TEST IN MOTHERS AND NEWBORN INFANTS

NUMBER OF CASES	DIAGNOSIS	NEGATIVE 0	1 PLUS	POSITIVE 2 PLUS	3 PLUS	4 PLUS	% NEGATIVE	% POSITIVE
15	Newborn infants (jaundiced)	1	4	7	3	0	33.3	66.7
11	Newborn infants (not jaundiced)	4	2	2	3	0	54.6	45.4
9	Normal pregnancy (six to nine months gestation)	9	0	0	0	0	100.0	0
65	Normal pregnancy (at term plus or minus one or two days)	37	5	10	12	1	64.6	35.4

newborn infants also brought about flocculation of the emulsion almost as frequently (45.4 per cent). In all these infants, the flocculation test became negactive in from seven to ten days. The sera of approximately one-third of normal pregnant women produced flocculation of the emulsion from twenty-four to forty-eight hours prior to delivery and continued to do so for from seven to ten days following parturition. In twelve of thirteen instances studied, the degree of positivity in mother and infant ran a parallel course. More detailed information concerning this phenomenon has been reported elsewhere.[15]

DISCUSSION

The data presented are in general agreement with those published by other investigators and therefore confirm further the usefulness of the cephalin-cholesterol flocculation test (Table VII). Its maximum value is apparent in

acute hepatitis, catarrhal jaundice, cirrhosis, or any other diffuse parenchymatous disease of the liver. A negative reaction, however, does not exclude one of these lesions. Similarly a positive flocculation cannot be interpreted as necessarily indicating liver dysfunction in the presence of other systemic disease.[1, 6, 11] As with flocculation tests for syphilis, further observation may clarify those situations in which "false positives" may be encountered. The data presented suggest that in the presence of infection, or during the puerperium, a positive test may be encountered without other evidences of liver dysfunction.

TABLE VII

COMPARISON OF PERCENTAGE OF POSITIVE CEPHALIN-CHOLESTEROL FLOCCULATION TESTS OBTAINED BY VARIOUS INVESTIGATORS

	CLAY AND MOORE[1]	HANGER[3, 4]	KIRSCHNER AND GLICKMAN[5]	LIPPMAN AND BAKST[6]	NADLER[10]	POHLE AND STEWART[11]	ROSENBERG[13, 14]	WADE AND GUTMAN
Acute hepatitis	----	----	65.5	----	100.0	94.4	100.0	100.0
Catarrhal jaundice	100.0	100.0	----	100.0	100.0	----	----	96.9
Cirrhosis	95.0	78.3	70.6	83.4	----	100.0	81.8	98.4
Congestive failure	20.0	0	----	40.0	----	37.9	22.3	44.7
Hemolytic icterus	----	0	33.3	----	----	----	----	50.0
Normal	0.2	0	----	4.8	----	0	----	0
Obstructive jaundice	----	11.7	17.3	22.1	3.6	78.3	----	48.7

Indeed, the purely empirical nature of the cephalin-cholesterol flocculation test is one of its chief disadvantages. Hanger believes the mechanism of the test to be similar to that of the flocculation tests for syphilis and to be dependent upon an altered globulin component.[3, 4] Quantitative as well as qualitative changes in the serum proteins encountered in nephrosis, sarcoidosis, chronic empyema, etc., are not necessarily associated with flocculation. The parallel course run by the flocculation test in mother and newborn infant suggests that the responsible agent does pass the placental barrier. The occurrence of positive tests in the presence of infectious disease as well as in the presence of allergic manifestations* lends some credence to Hanger's hypothesis. Until some definite rationale can be established, the test will probably be of limited usefulness.

Failure to understand the mechanism of the test could readily be forgiven if accurate distinction between obstructive and nonobstructive jaundice were possible, as has been claimed by several observers.[3-5, 14] The experiences in this laboratory are more akin to those of Pohle and Stewart,[11] who were unable to confirm this contention. It is indeed reasonable to expect a negative reaction until such time as obstruction can bring about secondary liver damage, but strongly positive flocculations have been encountered during the early phases of such obstruction and negative reactions after many years of repeated and severe bouts of obstructive disease. Sera of 48.7 per cent of the patients with obstructive jaundice caused flocculation of the emulsion, while the sera of 84.3 per cent of the patients with nonobstructive jaundice produced a positive re-

*Positive reactions have been encountered in the presence of asthma and urticaria, but too few instances in which liver disease can be excluded have been studied to justify description in this report.

action. Obviously one could not argue convincingly in any single instance concerning the nature of the jaundice.

Variations in the degree of positivity which occurred during the course of catarrhal jaundice as well as during the treatment of cirrhosis tend to confirm the claim that the flocculation test is of prognostic value.[3, 4, 14] These data will be reported elsewhere.

Conflicting reports have been published concerning the correlation of the cephalin-cholesterol flocculation test with other liver function tests.[4, 11] As is to be expected, however, one test appears more useful in one situation than does another. No exact correlation is demonstrable. The bromsulfalein test (Mateer's modification) in the absence of jaundice gives the most consistent evidence of liver disease. The intravenous hippuric acid and cephalin-cholesterol flocculation tests are next most reliable. The serum diastase test is least valuable of the tests examined.

CONCLUSIONS

1. The cephalin-cholesterol flocculation test is positive in 97.8 per cent of patients with diffuse parenchymatous liver disease.

2. Lesser percentages of positive reactions are encountered in focal disease of the liver.

3. Negative reactions may be encountered in the presence of minimal lesions but usually indicate the absence of hepatic dysfunction.

4. "False positives" occur during the course of infections, in the presence of allergic disease, or during the puerperium or the neonatal period.

5. No particular value can be ascribed to the test in the differentiation of obstructive from nonobstructive jaundice.

6. Quantitative changes in the globulin have no apparent role in determining the occurrence of positive reactions.

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THE VALUE OF THE WELTMANN SERUM COAGULATION REACTION FOR THE DIAGNOSIS OF CERTAIN FORMS OF MALIGNANT NEOPLASTIC DISEASE

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WELTMANN, in 1930,[1, 2] described a simple serum reaction which shows characteristic changes in various pathologic clinical conditions. If normal blood serum is diluted fifty times with a solution of $CaCl_2 \cdot 6\ H_2O$, the concentration of which ranges from 0.1 to 0.01 Gm. per 100 c.c., and is then boiled for fifteen minutes, it will coagulate in those tubes containing the 0.1 to 0.4 or .05 per cent concentrations (first 6 or 7 tubes). This constitutes a normal serum coagulation reaction or a normal coagulation band. Under abnormal conditions coagulation may occur in fewer than 6 test tubes (the coagulation band is shortened or shifted to the left) or there may be coagulation in more than 7 test tubes (the coagulation band is lengthened or shifted to the right). Shortening of the coagulation band is found in exudative inflammation or in processes which lead to tissue necrosis in the body. The change is independent of the cause of this necrosis. It is also found in nephrosis. Lengthening of the coagulation band is found in processes which lead to fibrosis, particularly cirrhosis of the liver, and in liver damage, hemolytic anemias, and septicemias.

The present investigation was undertaken to determine whether this reaction could help in the detection of malignant neoplastic disease in the body and whether it could help differentiate between similar clinical conditions; for example, between a new growth and other pathologic changes. Theoretically, the coagulation band would not be changed by the presence of the tumor itself but would be altered by regressive or inflammatory changes present in the tumor tissue.

Method.—The Weltmann test was performed according to Weltmann's original method. It is, however, advantageous to use calcium chloride containing 2 molecules of water of hydration as suggested by Dees[3] instead of the very hygroscopic product containing 6 molecules of water.[4] A lengthening of the coagulation band to tube 7½ is abnormal, although it has been shown that this might occasionally occur in old people without obvious reason.[5] Sera from normal individuals show coagulation in 6 to 7 test tubes, as stated by Weltmann and many other investigators. Kraemer,[6] however, found that sera from normal individuals gave a coagulation band of from 5 to 7 tubes. Although the presence of some kind of infection is the probable reason for the value of 5 in apparently normal individuals, we shall in this paper designate a coagulation from 0 to 4 as significantly shortened; a coagulation of 5 is regarded as being suggestively shortened.

Results.—Sera of 128 patients with definite evidence of malignant neoplastic disease were examined. In Table I the cases are grouped according to

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the organs in which the neoplasm originated. Exception is made for the last three groups which include malignant tumors combined with obstructive jaundice, malignant tumors in which ascites was the predominant clinical feature, and malignant tumors in which involvement of the bones was present.

In the control group, sera from 300 patients with various diseases were examined. Many of these sera were taken from patients who had lesions which clinically were similar to malignant neoplastic lesions; for example, fibrosis of breast tissue, fibromyomas of uterus, etc.

TABLE I

ORGAN	NUMBER OF CASES	COAGULATION IN NUMBER OF TEST TUBES								
		0	1 (0.067 % $CaCl_2 \cdot 2H_2O$)	2 (0.0603 % $CaCl_2 \cdot 2H_2O$)	3 (0.0536 % $CaCl_2 \cdot 2H_2O$)	4 (0.0469 % $CaCl_2 \cdot 2H_2O$)	5 (0.0402 % $CaCl_2 \cdot 2H_2O$)	6 (0.0335 % $CaCl_2 \cdot 2H_2O$)	7 (0.0268 % $CaCl_2 \cdot 2H_2O$)	7½ (0.0234 % $CaCl_2 \cdot 2H_2O$)
Brain	1					1				
Larynx	1			1						
Thyroid	1					1				
Lung	8	1		1	1	1	3	1		
Uterus	3					2	1			
Cervix	4				1		2		1	
Ovary	2	1		1						
Kidney	4			1	1	1	1			
Esophagus	4				1	2	1			
Stomach	16	1		5	1	2	6	1		
Ileum	2				2					
Colon	19	2	1	3	3	3	3	1	3	
Rectum	11		1	1		4	4	1		
Breast	9			1	1	1	3	1	2	
Gall bladder	1							1		
Carcinomatosis and ascites	11	5	1	1	2	1			1	
Cases with bone involvement	12	8[4]	1	1		1	1			
Cases with obstructive jaundice	19					3	4	5	3	4
Total number	128	18	4	16	13	23	29	10	11	4

Among the 128 patients suffering from malignant neoplastic disease, twenty-one (16.4 per cent) showed a normal coagulation band, four (3.1 per cent) a lengthened, twenty-nine (22.7 per cent) a suggestive, and seventy-four (57.8 per cent) a markedly shortened coagulation band.

Among the nineteen patients in whom obstructive jaundice was the prominent clinical feature, ten had carcinoma of the pancreas and three each had carcinoma of the gall bladder, stomach, or bile ducts. Only three of these patients had a coagulation band of 4 and four a coagulation band of 5; the others had a normal or slightly lengthened coagulation band. Jaundice and hepatic damage tend to lengthen the coagulation band and to counterbalance the tendency to the left shift which might be initiated by the presence of the tumor. The coagulation band in these patients is therefore frequently normal or slightly lengthened. If in a case of jaundice the coagulation band is shortened, diffuse hepatitis, which shows marked right shift of the coagulation band, can be excluded with a good degree of probability. No help, however, is gained by the test concerning the etiology of the obstructive jaundice, since inflammatory changes may accompany obstructive jaundice caused by gallstones as well as by carcinoma.[4] If the jaundice cases are excluded, a left shift in the coagula-

tion band was found in about 88 per cent of 109 sera. Shortening was marked in seventy-one sera (65.2 per cent). The value of the reaction in the diagnosis of malignant neoplastic disease, however, is markedly diminished if the differential diagnosis of diseases which resemble each other clinically is considered. In patients with infiltrative lesions in the lung, the coagulation band will be shortened regardless of the underlying disease, whether carcinoma, exudative tuberculosis, or pneumonitis. The coagulation band, however, will be lengthened if the infiltration is due to a fibrosing process in the lungs. The reaction is of little diagnostic help in carcinoma of the breast and the female genital organs, since patients with inflammatory lesions or benign tumors, that is, fibromyomas with necrosis, may show a left shift in the coagulation band. This is equally true for the differentiation between benign fibroadenomatous and carcinomatous enlargement of the prostate. In carcinomas of the gastrointestinal tract, however, a positive reaction is of diagnostic value. In patients with symptoms referable to the esophagus, a shortened coagulation band favors the diagnosis of an organic lesion as opposed to spasm. If disease of the stomach is suspected, a shortening of the coagulation band suggests the presence of carcinoma or ulcer in an active stage. In cases of hematemesis a shortened coagulation band is evidence against esophageal varices as the cause of the gastric bleeding, since in the most common disease leading to this complication, cirrhosis of the liver, there is usually a lengthened coagulation band. In the presence of hyperchromic anemia, a normal or lengthened coagulation band supports the diagnosis of pernicious anemia, while a shortened coagulation band points to a complicating carcinoma.

If the bones are involved by neoplastic disease, very marked shortening of the coagulation band is found. Among twelve patients, five primary tumors were in the prostate, three in the breast, one in the kidney, and one in the cervix; one patient had multiple myeloma, and in another the primary site of the carcinoma could not be determined. We found a Weltmann reaction of 0 in eight of nine patients in whom there was marked involvement of the bones.

The coagulation band was found to be of aid in the differential diagnosis of conditions associated with ascites. If in a case of this kind the ascites is not a complication of renal or cardiac disease or due to tuberculosis, the most important differential diagnosis rests between carcinomatosis of the peritoneum and cirrhosis of the liver. Of eleven patients examined, ascites was due to carcinomatosis of the ovary in ten and to an undetermined neoplasm of the intestinal tract in one. The coagulation band was 0 five times, 1 to 4 five times, and normal only once. In a case of subleucemic lymphatic leucemia, in which ascites was the leading clinical symptom, the coagulation band was 4. This was the first laboratory examination which lead to reconsideration of the diagnosis of cirrhosis of the liver. In cirrhosis of the liver, on the other hand, the coagulation band is usually lengthened. In nine patients in whom ascites was the predominant clinical symptom, we found coagulation bands of 8 four times, of 7½ twice, and of 7 twice; only once, in a case complicated by severe inflammatory pelvic disease, proved by autopsy, was a coagulation band of 5 or 6 found.

DISCUSSION

The diagnostic and prognostic significance of changes in the coagulation band in different diseases has been established in a great number of cases by various authors.[4-13, 15-22]

The coagulation band is a nonspecific reaction. Both lobar pneumonia and nephrosis, for instance, will lead to a marked left shift. The test is useful, therefore, only when certain special diagnostic or prognostic problems are involved. This has been stressed very strongly by Teufl[7] and Levinson and Klein.[11] Two diseases present at the same time may influence the coagulation band in the same direction or counterbalance each other. It is therefore, necessary to define the differential diagnostic problem before employing the test. If this is done, it can be of considerable diagnostic value.

The changes of the coagulation band in patients having malignant neoplastic diseases have been investigated by several authors. Some[23, 24, 25] did not find significant changes, while others[7, 12, 13, 16, 26-29] found a left shift in many of the patients examined. Our results agree well with this latter result, since 65 per cent of our patients with carcinoma not complicated by obstructive jaundice showed a significant shift and an additional 23 per cent a suggestive shift to the left. The test may be applied, therefore, as a diagnostic procedure when the presence of malignant neoplastic disease is suspected. This is in full agreement with Kraemer,[6] who feels that any patient having a coagulation band of 4 or less has either an exudative inflammatory disease or a malignant neoplastic process. The test is very valuable in the differentiation of organic disease from functional nervous disturbance. If, however, the clinical examination reveals the presence of a lesion in lungs, breast, female genital organs, or prostate, little information is gained by this reaction, since inflammatory lesions cause the same degree of shortening of the coagulation band. Similar findings have been reported by Ottsen.[28] The coagulation band is of value in the diagnosis of malignancies of the stomach, especially if a pernicious anemia-like blood picture or hematemesis is the leading clinical manifestation. It is frequently of diagnostic significance in malignancies of the esophagus, as well as of the small and large intestines. The Weltmann test is of great value in the differential diagnosis in patients in whom ascites is present provided that cardiac, renal, and tuberculous disease can be excluded. Finally, in those in whom diffuse neoplastic involvement of the bones is present, the coagulation band is markedly shortened. Occasionally such shortening may be found in diseases of the bone not due to neoplastic malignant disease, for example, Paget's disease with pathologic fracture, but these are exceptions and are comparatively rare. The behavior of the coagulation band in multiple myeloma deserves some comment. In one instance we found a left shift to 0. Similar results have been reported in the literature.[12] Fleischhacker,[30] on the other hand, described a lengthening of the coagulation band to 10 test tubes as characteristic for this disease.

A satisfactory explanation for the mechanism of this reaction was lacking until recently.[3] Definite progress has been made since the analysis of the serum protein fractions has been made possible by the electrophoretic procedure of Tiselius. Changes in the gamma globulin were found to be responsible for the positive result in the colloidal gold reaction in blood and liquor and for the positive cephalin flocculation test.[31-33] Scherlis and Levis[21, 34] have recently shown that sera containing much alpha globulin usually have a shortened coagulation band and those with a small amount of alpha globulin usually have a prolonged coagulation band. Thus it appears probable that changes occurring in the qualitative composition of the serum proteins produce the altered colloid stability of the serum.

SUMMARY

The Weltmann serum coagulation reaction shows a marked left shift in the large majority of patients with malignant neoplasm. It is suggested, therefore, that the test has differential diagnostic value in selected cases of suspected malignant neoplastic disease. This is particularly true when the lesion is in the gastrointestinal tract. When, however, the lesions are in the lungs, breast, female genital organs, or the prostate, the test is of little help in differentiating malignant from similar benign neoplastic or inflammatory conditions. When ascites exists and is not a complication of previous cardiac, renal, or tuberculous disease, a shortened coagulation band is suggestive of carcinomatosis, while a lengthened coagulation band is suggestive of cirrhosis of the liver. A very marked shortening of the coagulation band is also found in the majority of patients in whom neoplastic involvement of the bones is present. An unexpected shortened coagulation band, not explained by the clinical findings, should always suggest that a thorough examination of the skeleton be made.

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STUDIES OF THE PHARMACOLOGY OF ATABRINE* ON MICE, RATS, DUCKS, AND DOGS

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Hecht[1] and Dawson and co-workers[2] reported results of studies of the toxicity of Atabrine dihydrochloride (Quinacrine Hydrochloride, U.S.P.) for the mouse, rabbit, and cat. Similar studies were made on the dog by Martin and co-workers[3] and Clark and associates.[4] Hecht[5] also reported extensive pharmacologic data on laboratory animals. These included the effects on the central nervous system, the heart and circulation, and the smooth muscle structures, as well as information on the absorption, distribution, and routes of excretion after medication. The growth-inhibiting and pathologic effects of chronic Atabrine medication on young growing rats were also studied by Wright and Lillie.[6]

On the basis of available literature, it appears that Atabrine is rapidly destroyed or chemically modified in the organism, and storage occurs to a limited extent only. Dearborn and co-workers[7] observed, following chronic daily oral medication of dogs with Atabrine, that after the second week of medication neither elimination nor storage of the Atabrine increased and an average of less than 4 per cent was eliminated in the urine and feces. Annegers and associates[8] recently published results of studies on the retention and excretion of Atabrine in the rat and dog after chronic medication, with particular attention to the bile and urine and cholic acid output. The urinary excretion in the dog was from 0.5 to 5.0 per cent of the daily dosage administered. The biliary excretion did not exceed 82 per cent of doses, some of which were two and one-half times the dose for human beings on a body weight basis.

The entry of the United States into World War II created a demand for large supplies of Atabrine of American manufacture. Some question arose on the basis of the initial experience in the Armed Forces and whether the American product was equivalent to that manufactured in Germany. A study of this subject, recently reported by Loughlin and co-workers,[9] proved the clinicial indistinguishability of the two products.

The opportunity presented itself in this laboratory to investigate the question further, using Atabrine prepared by the American process from both American and German intermediates with Atabrine prepared by the German process from German intermediates. These preparations of Atabrine were compared on rats, ducks, and dogs. In addition, much other pertinent data on the toxicity of Atabrine to mice, rats, rabbits, ducks, and dogs have been obtained and are being reported in this publication.

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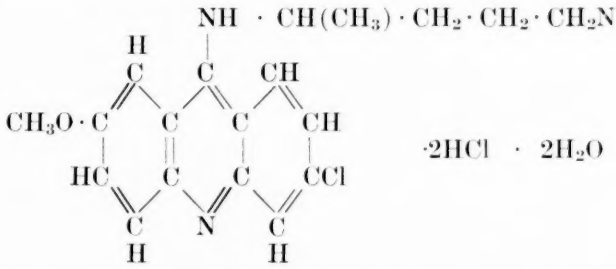
Chemistry.—Atabrine (quinacrine) is 3-chloro-7-methoxy-9-(1-methyl-4-diethylamino)butylamino-acridine which is customarily used in the form of the dihydrochloride. The structural formula is as follows:



Description.—Atabrine dihydrochloride is an odorless, bitter-tasting, orange-yellow, crystalline powder, containing 2 mol. of water of crystallization and melts at from 245 to 247° C. with decomposition. The anhydrous salt has a melting point of from 253 to 256° C. The solubility in water is 3.5 per cent at 20° C.; it is slightly soluble in acetone, ether, and benzene. Aqueous solutions are yellow in color and exhibit a yellow-green fluorescence. The characteristics of the dihydrochloride and other salts of Atabrine are summarized in Table I. The dimethane sulfonate is also known as the "musonate."

TABLE I

CHARACTERISTICS OF EIGHT ATABRINE SALTS

SALT	FORMULA R = ATABRINE BASE	DRIED SALT (MOL. WT.)	DRIED SALT (M.P. °C.)	pH OF 2% SOLUTION	SOLUBILITY IN H_2O AT 25° C. (GM./100 GM. SOLUTION)	PER CENT HYDROLYSIS (2 HR. AT 100° C.)
Dihydrochloride	R·2 HCl	472.5	253-256	4.50	4.2	13.1
Dimethane sulfonate	R·2 CH_3SO_3H	591.5	136-138	4.60	59.3	14.9
Dilactate	R·2 $CH_3CHOH·COOH$	579.5	115-118	6.30	61.4	27.6
Disulfamate	R·2 NH_2SO_3H	593.5	151-153	3.95	33.0	12.2
Tartrate	R·$COOH(CHOH)_2COOH$	549.5	139-141	4.50	9.6	17.3
Citrate	R·$C_6H_8O_7$	591.5	130-135	5.00	33.1	21.6
Di-(acetyl salicylate)	R·2 $C_6H_4(COOH)OCOCH_3$	759.5	>300 (dec.)	5.85	7.6	14.0
Diformate	R·2 HCOOH	491.5	88-93	4.00	45.8	13.2

Mietzsch and associates[10] determined the stability of solutions of Atabrine dihydrochloride and observed that aqueous solutions gradually decomposed by hydrolysis due to splitting off the long aliphatic side chain. Although these authors found no deterioration in a 1 per cent solution after three weeks' aging, they did observe that sterilization accelerated hydrolysis. In view of this, only freshly prepared solutions (up to twelve hours) were used in this study.

Acute Toxicity of Atabrine for Albino Mice.—Table II presents data obtained in determining in mice the acute toxicity of Atabrine (the free base as well as four salts of Atabrine) and quinine when given by mouth. The drug was given through a stomach tube in appropriate volumes of solution. Experiments were carried out similarly in which the four Atabrine salts were given sub-

TABLE II

ACUTE TOXICITY TO MICE OF QUININE AND OF ATABRINE AND FOUR OF ITS SALTS

COMPOUND	FORM	LD_{50} ± S.E.,* (GM./KG.) ACCORDING TO ROUTE OF ADMINISTRATION			
		ORAL	SUBCUTANEOUS	INTRAPERITONEAL	INTRAVENOUS
Quinine	Hydrochloride	0.880 ± 0.017	—	—	—
Atabrine	Base (in oil of sesame)	0.755 ± 0.006	—	—	—
Atabrine	Base	0.838 ± 0.023	—	—	—
Atabrine	Dihydrochloride	0.706 ± 0.016	0.840 ± 0.016	0.194 ± 0.008	0.020 ± 0.001
Atabrine	Disulfamate	—	0.900 ± 0.009	0.258 ± 0.005	0.028 ± 0.001
Atabrine	Dilactate	—	0.980 ± 0.027	0.257 ± 0.004	0.035 ± 0.002
Atabrine	Dimethane sulfonate	—	0.890 ± 0.011	0.218 ± 0.003	0.028 ± 0.002

*s.e. indicates the approximate standard error of the LD_{50}, estimated graphically[11] and expressed in the same units as the LD_{50}.

cutaneously, intraperitoneally, and intravenously. The tests were conducted on male albino mice weighing between 16 and 32 Gm. Most of the LD_{50} values given in Table II represent data taken on groups of from thirty to fifty mice, although as many as ninety-six mice were used in one determination. With the one exception indicated in Table II, all of the preparations were either dissolved or suspended in water. The actual levels of toxicity are clearly shown in Table II and need not be discussed in detail inasmuch as they confirm and extend the data previously published.[1, 2] It is noteworthy, however, that the dihydrochloride is more toxic by mouth than by subcutaneous administration. Also, for a given route of parenteral administration, the LD_{50} of the four forms tested is approximately the same, and a ratio of 32:8:1 holds generally for the subcutaneous, intraperitoneal, and intravenous routes, respectively.

Toxicologic Comparison of Atabrine Manufactured by American and German Processes.—Specimens of pharmaceutic grade Atabrine prepared by three of the four possible combinations of intermediates and processes of manufacture were administered to young adult albino rats to determine whether there was any difference in the acute oral toxicity. The data given in Table III indicate that the LD_{50} is the same for the three preparations. Atabrine manufactured by the American process from American intermediates was given both as the free base and as the dihydrochloride. The free base proved to be slightly less toxic, but the difference cannot be considered significant in view of the respec-

TABLE III

ORAL TOLERANCE OF THE ALBINO RAT (140 TO 190 GM.) TO ATABRINE OF AMERICAN AND GERMAN MANUFACTURE

PREPARATION	PROCESS	FORM*	NUMBER OF RATS	LD_{50} ± S.E. (GM./KG.)
1	American process with American intermediates	Base	50	0.670 ± 0.026
2	American process with American intermediates	Dihydrochloride	64	0.628 ± 0.030
3	German process with German intermediates	Dihydrochloride	80	0.640 ± 0.021
4	American process with German intermediates	Dihydrochloride	60	0.615 ± 0.037
5	Combination of preparations 2, 3, and 4	Dihydrochloride	204	0.615 ± 0.015

*All dosages were given on basis of anhydrous compound.

tive standard errors. There is no question of a difference in toxicity in the three samples given in the form of the dihydrochloride nor of the equality of their toxicity with that of a combination of all three.

The three specimens of Atabrine were administered orally to dogs, six dogs receiving each specimen. The dogs weighed from 8 to 21 kg. and were given 25 mg. of Atabrine per kilogram of body weight three hours prior to feeding on each of three successive days. The animals were carefully watched for three hours after dosing for the development of symptoms of intolerance and to observe whether the drug was retained. All of the animals appeared to be nauseated to some extent and Dogs 5 and 12 vomited after eating on two days. The dogs suffered from a slight to moderate anorexia so that a 5 to 10 per cent weight loss occurred due to a decreased food and water intake. Each of the animals was sacrificed twenty-four hours after the last dosage and autopsied. No gross or histologic abnormalities due to medication were demonstrable. The heart, liver, lungs, spleen, and kidneys were removed and analyzed for Atabrine. The analytic data obtained are presented in Table IV.

Included in Table IV are the data on the two dogs which vomited part of the Atabrine given. A third dog (Dog 4) showed such marked diarrhea that absorption must have been impaired. Since it is probable that this accounts for the relatively low Atabrine content in the various tissues analyzed, these data have not been included in the averages.

The analytical data present several points of interest. It is clear that the three different lots of Atabrine are stored to the same extent in all the organs examined. Hence the amounts of Atabrine recovered in these organs, expressed as the percentages of the total dose given, are the same for all three lots. The average recovery in the whole series, with the exceptions noted above, was 17 per cent of the dose given in the three-day course of medication. The comparative affinity of liver tissue for Atabrine is well demonstrated. Three-fourths of the drug recovered was found in this organ, thereby demonstrating strikingly that the liver is the body's principal depot for Atabrine.

In addition to the acute experiments reported on rats and dogs, a growth experiment was carried out on ducks. The duck regurgitates substances having a local irritant action or a disagreeable taste. As a consequence, the LD_{50} in acute experiments could not be determined on normal ducks, and the possibility of ligating the esophagus was not explored. To determine the effect on growth, groups of ten or eleven young Pekin ducks, averaging 100 Gm. in weight, were segregated in false-bottomed cages and were fed a growth mash diet in which was incorporated a concentration of 0.005, 0.025, or 0.125 per cent (calculated as the anhydrous compound) of the three specimens of Atabrine. The two lower dosages closely approximate the dietary drug levels used in routine screening tests of Atabrine against experimental *Plasmodium lophurae* infections in the duck and represent the levels which are effective from a therapeutic and curative standpoint. The medication was continued for thirty-five days. For purposes of comparison, quinine sulfate was given to similar groups of ducks at levels comparable from the chemotherapeutic standpoint.

Figs. 1, 2, and 3 represent the average weights of the ducks throughout the experiment. The growth curves for the three different Atabrine specimens

TABLE IV

DISTRIBUTION OF ATABRINE FOLLOWING ORAL ADMINISTRATION TO DOGS

ATABRINE DIHYDRO-CHLORIDE	DOG	TOTAL DOSE (MG.)	HEART		LUNGS		LIVER		SPLEEN		KIDNEYS		ATABRINE RECOVERED (% OF TOTAL DOSE)
			A	B	A	B	A	B	A	B	A	B	
American	12	825	1.3	0.18	4.1	0.50	8.2	4.54	5.1	0.23	1.1	0.09	5.5*
process with	14	1523	4.1	0.39	15.6	2.14	35.4	12.20	27.0	1.08	5.8	0.35	16.2
German	15	578	2.9	0.40	12.5	1.90	38.6	16.70	18.7	0.55	5.8	0.48	20.2
intermediates	60	675	3.7	0.33	17.5	2.50	33.6	15.70	17.1	0.69	15.0	0.91	20.0
	23	950	1.5	0.15	7.6	1.10	10.0	6.00	10.3	0.33	3.8	0.29	8.0
	16	600	5.5	0.67	9.1	1.30	38.0	15.30	21.8	0.91	7.0	0.52	19.0
Average			3.6	0.39	12.5	1.79	31.1	13.20	19.0	0.71	7.5	0.51	16.7
American	2	728	3.8	0.41	12.3	1.42	21.5	10.20	11.7	1.05	15.2	1.15	14.2
process with	4	930	1.6	0.18	11.0	1.32	6.3	2.70	6.2	0.93	3.5	0.23	5.3*
American	5	967	1.6	0.18	10.9	1.66	30.2	13.50	14.0	0.57	3.7	0.29	16.2
intermediates	6	1013	1.4	0.17	2.8	0.34	7.6	5.50	4.0	0.27	1.8	0.16	6.5*
	9	1088	5.2	0.45	16.0	1.71	30.0	11.80	28.8	0.69	5.0	0.34	15.0
	40	555	3.8	0.38	16.3	2.30	32.2	11.90	20.0	1.10	7.1	0.45	16.0
Average			3.6	0.36	13.9	1.77	28.5	11.85	18.6	0.85	7.8	0.56	15.4
German	7	645	5.8	0.59	13.6	1.45	52.5	16.00	20.4	0.95	5.8	0.37	19.4
process with	13	1440	5.1	0.63	11.5	1.51	46.3	19.30	18.3	0.58	13.3	0.92	22.9
German	17	763	2.9	0.24	9.3	1.30	20.4	7.50	25.5	0.47	6.9	0.45	10.0
intermediates	1	645	2.4	0.21	8.8	1.10	15.9	7.35	9.5	0.21	1.8	0.14	9.0
	22	833	6.2	0.67	12.3	1.70	32.3	16.90	17.1	0.60	8.3	0.65	20.0
	21	675	6.7	0.73	22.1	4.40	46.1	21.20	21.8	0.74	14.0	1.10	28.0
Average			4.8	0.51	12.9	1.91	35.6	14.71	18.8	0.59	8.4	0.60	18.2
Average—all			4.0	0.42	13.1	1.82	31.7	13.25	18.8	0.72	7.9	0.56	16.8

A = Atabrine—mg. per 100 Gm. of organ.
B = Per cent of total dose given.
*Dogs 6 an 12 showed emesis after two of the doses and Dog 4 showed severe diarrhea; these values have been omitted from the averages.

are indistinguishable on the two lower dosage levels. The growth curves for the groups receiving 0.125 per cent Atabrine indicate that the specimens prepared from the imported intermediates were tolerated less satisfactorily than that from domestic sources. Only two deaths resulted in the group of ten ducks receiving the latter at this level, indicating further that this product was significantly less toxic than the Atabrine prepared by either process from imported intermediates. The odds are more than 100:1 against observing by chance six or more deaths in a group of ten when the true expectancy is only two. As may be noted from Figs. 1 and 2, six and eight deaths, respectively, were observed in the two groups receiving Atabrine from imported intermediates.

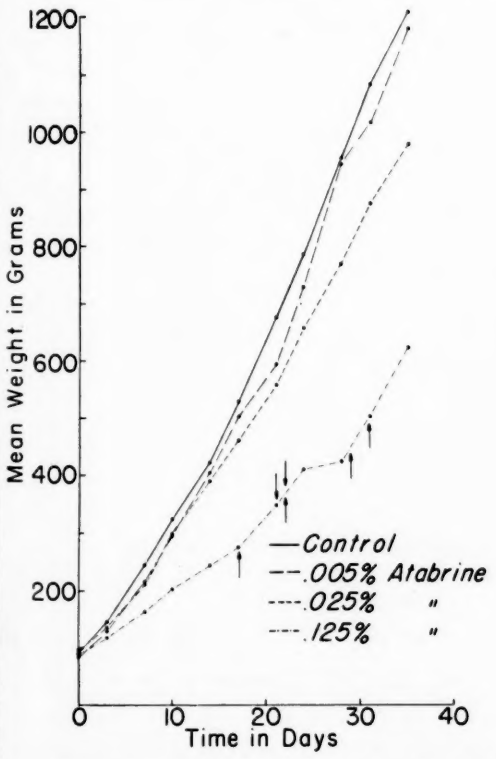


Fig 1.—Influence of Atabrine on the growth of ducks; German process with German intermediates.

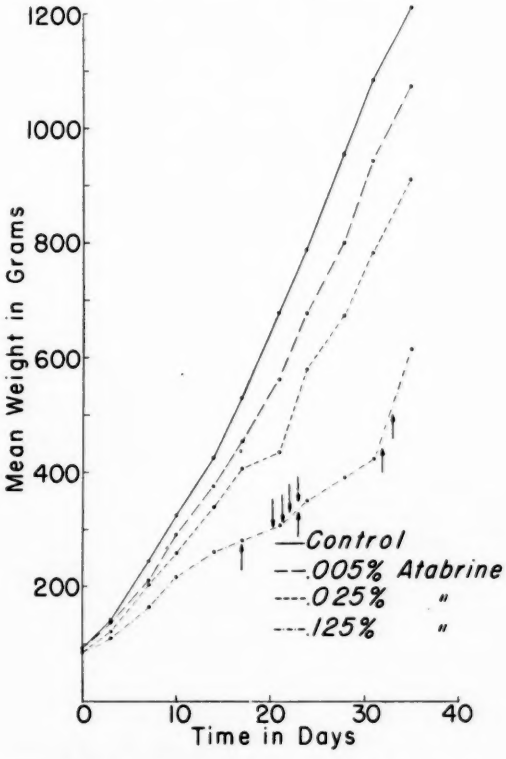


Fig. 2.—Influence of Atabrine on the growth of ducks; American process with German intermediates.

The results obtained in the parallel quinine sulfate growth test are presented in Fig. 4. Since on a weight basis quinine is only one-half as effective as Atabrine in clearing experimental *Plasmodium lophurae* infections in ducks, the concentrations of quinine used were twice those chosen in the Atabrine diets. The growth curves for the three levels of quinine agree very well with the corresponding curves for the Atabrine of American process and American intermediates, except that two deaths occurred on the highest concentration of the Atabrine.

The toxicologic data thus obtained on three species of animals show conclusively that if there is any demonstrable difference in these specimens of

Atabrine, the advantage is in favor of that manufactured by the American process from American intermediates.

The Deposition and Retention of Atabrine in the Tissues of the Rat.—To determine the distribution of Atabrine deposited following a single large dose, male rats weighing from 400 to 450 Gm. were fasted eighteen hours and then given 675 mg. per kilogram of body weight by stomach tube. The survivors, constituting about 40 per cent of those given this dose, were sacrificed in groups of three to nine rats at 4, 8, 24, 48, 72, 120, and 240 hours after dosing. The organs and gastrointestinal contents listed in Table V were removed and analyzed for their Atabrine content. In actual amount, the liver showed more

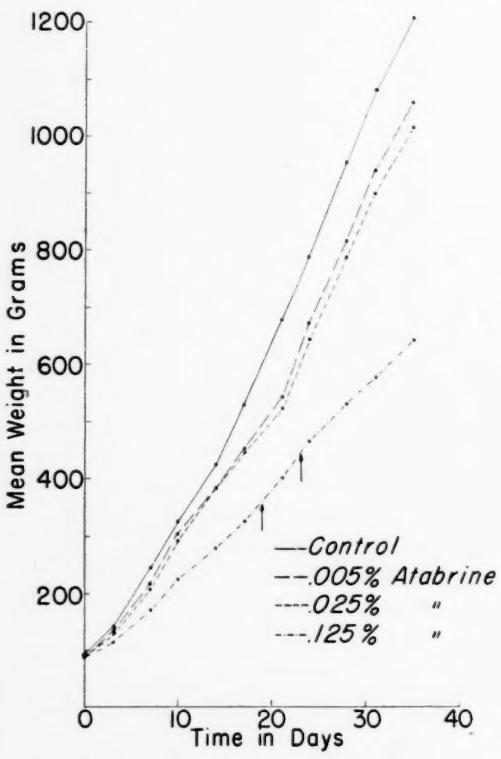


Fig. 3.—Influence of Atabrine on the growth of ducks; American process with American intermediates.

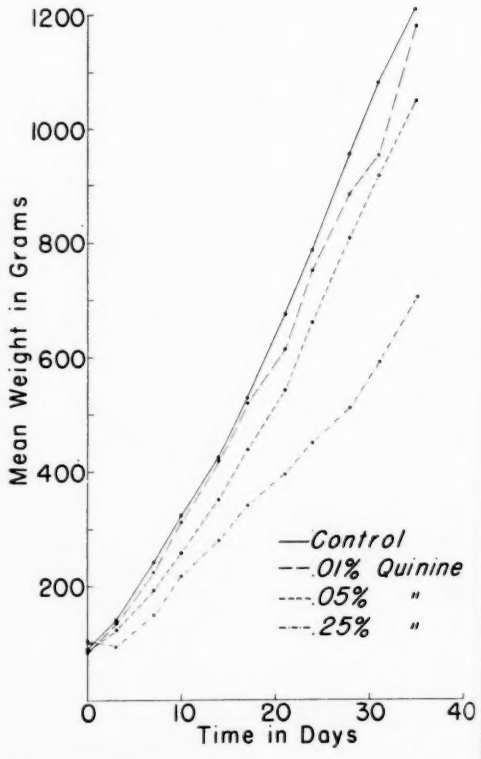


Fig. 4.—Influence of quinine on the growth of ducks.

than that found in all the other organs examined; the total amount recovered, however, was but a fraction of that given. The data indicate that the Atabrine concentration of the liver and spleen increases during the first eight to twenty-four hours and then decreases. The rate of disappearance is not the same for all organs, the indication being either that the mobilization of the deposits in the liver and spleen tends to keep the other tissues saturated or that the latter have a greater relative affinity for the drug.

Deposition of Atabrine in Various Organs During and After Chronic Medication.—To determine the degree of Atabrine deposition, without disturbing the course of the experiments illustrated in Fig. 1, a second experiment was

TABLE V

AMOUNT AND CONCENTRATION OF ATABRINE IN RATS AFTER A SINGLE DOSE OF 675 MG. PER KILOGRAM ORALLY.

ORGANS	AMOUNT (MG.)							CONCENTRATION (MG./GM.) OF TISSUE OR GASTROINTESTINAL CONTENTS						
	HOURS AFTER MEDICATION							HOURS AFTER MEDICATION						
	4	8	24	48	72	120	240	4	8	24	48	72	120	240
Liver	1.81	2.45	1.65	1.31	0.70	0.73	0.52	0.13	0.16	0.10	0.08	0.04	0.04	0.03
Kidneys	0.22	0.19	0.26	0.18	0.14	0.11	0.04	0.07	0.07	0.07	0.06	0.05	0.04	0.01
Lungs	0.21	0.19	0.18	0.12	0.12	0.10	0.03	0.08	0.08	0.06	0.05	0.04	0.03	0.01
Spleen	0.39	0.01	0.13	0.10	0.06	0.06	0.03	0.09	0.16	0.22	0.19	0.12	0.09	0.04
Heart	0.04	0.04	0.04	0.02	0.02			0.02	0.03	0.03	0.02	0.02		
Stomach (empty)	0.04	0.04	0.04	0.02	0.01			0.03	0.02	0.03	0.01	Trace		
Stomach contents	2.06	0.80	0.75	0.06				0.42	0.22	0.22	0.04			
Intestinal contents	0.80	0.53	0.39	0.15				0.21	0.14	0.08	0.05			
Cecal contents	0.55	0.39	0.33	0.11				0.12	0.10	0.05	0.02			

set up, using from 45 to 50 Gm. male rats in groups of such size that from five to ten animals could be sacrificed at intervals after the daily drug dosage was discontinued. Three such groups of animals received Atabrine by stomach tube daily for forty-nine days in dosages corresponding respectively to 2.5, 5, and 10 per cent of the LD_{60} for rats of this age and weight. A fourth group received 20 per cent of the LD_{60} for only five days. The tissue Atabrine analyses were carried out by the procedure described by Hecht.[1] Blood concentrations were determined by a photofluorometric method.[12]

The results obtained by chemical analyses of blood and liver at the indicated intervals are illustrated as means in Table VI. The data indicate that a significant blood concentration was maintained in the rat as long as Atabrine deposits existed in the liver. These deposits disappeared rapidly and approached zero by from forty to sixty days after discontinuance of medication.

TABLE VI

MEAN ATABRINE CONCENTRATION OF BLOOD AND LIVER OF RATS

DAILY DOSE (MG./KG.)	DAYS OF MEDICATION	TOTAL DOSE (GM./KG.)	NUMBER OF RATS	DAY SACRIFICED	AVE. LIVER WT. (GM.)	LIVER ATABRINE (MG./GM.)	BLOOD ATABRINE (γ/100 C.C.)
22.5	49	1.10	8	50th	9.90	0.08	25
			8	64th	11.90	0.002	3
			8	78th	11.56	Trace	Negative
45.0	49	2.20	9	50th	9.15	1.62	50
			9	61st	10.63	0.05	5
			7	78th	10.90	0.026	0–10*
			7	90th	11.71	0.004	Negative
90.0	49	4.40	7	50th	12.17	4.58	300
			8	64th	9.64	2.00	30
			7	71st	11.45	0.82	26
			5	92nd	11.47	0.106	6
			9	106th	11.83	0.007	3
180.0	5	0.90	10	6th	2.45	1.28	580
			9	13th	3.26	0.62	260
			9	20th	4.86	0.24	Trace
			9	27th	6.16	0.016	Negative
			9	34th	6.36	0.004	Negative

*5, negative; 2, 5 to 10 γ/100 c.c.

Gross and microscopic examinations were made of the tissues of all animals in the chronic growth studies. The findings on necropsy of animals fed daily dosages of 5 per cent or less of the LD_{60} were insignificant. However, the following description was typical of the liver of those receiving 10 per cent of the LD_{60}.

Liver: Markedly enlarged, discolored, highly pigmented with Atabrine; lobules very prominent in from 25 to 30 per cent of the organ. The coloring shaded from dark reddish blue or greenish purple to dirty yellow at periphery. Two areas, 1.5 by 2 cm., were necrotic; adhesions to diaphragm, stomach, intestines, and right kidney. Large yellow masses were deposited in liver. In volume such deposits represented one-fifth to one-fourth of the entire organ.

These deposits represented necrotic liver tissue and Atabrine. However, analysis indicated that the Atabrine content of liver tissues of relatively normal gross appearance was not greatly different from that of the cheeselike yellow-green necrotic areas. The Atabrine content of grossly normal, but strongly colored, liver tissues from selected animals of those given 45 mg. per kilogram daily was 1.25 mg. per gram; the corresponding value from necrotic areas was 1.22 mg. per gram. Of those animals given daily 90 mg. of Atabrine per kilogram of body weight, the corresponding values were 2.33 and approximately 4.2 mg. per gram.

It appears, therefore, that Atabrine deposits in the liver of the rat under the conditions of these tests exist largely in the form of organic acid salts and, to a limited extent only, in the form of the base. The other typical pathologic changes were as follows:

Kidneys: Greatly enlarged, mottled with minute yellow granules, and deposition in tubules. Right kidney frequently adherent to liver. Left kidney free.

Adrenals: Enlarged about two to four times normal size and deeply pigmented with Atabrine.

Spleen: Very greatly enlarged. Yellow tinted.

Heart: Moderately enlarged and tinted yellow.

Lungs: Deep yellow tint.

Bones: Soft and yellow colored.

Gastrointestinal Tract: Deep yellow color.

Skin: Deep yellow color.

Such changes and the marked deposition of Atabrine just described largely disappeared from fifty to sixty days after discontinuance of heavy medication.

Influence of Chronic Medication with Large Doses of Atabrine on the Blood Picture.—Yuge[13] observed a marked increase in the reticulocyte count in twelve children within a week after the beginning of therapy of tropical malaria with Atabrine and Plasmochin. Steinberg[14] noted no morphologic or physicochemical changes in the blood after a more or less lengthy period of chronic medication with dosages within a therapeutic range. Large doses caused unimportant reductions in red cell counts and hemoglobin values, and hemolysis or formation of methemoglobin was not observed. Hecht confirmed the observations of Steinberg experimentally and also reported that the reticulo-

endothelial system of the normal dog, or of other animals, after piroplasmoses infections was unaffected by Atabrine.

Siegenbeek van Heukelom[15] observed that in acute tropical malaria, patients with hemoglobin values above 60 per cent showed a slight decrease and those with lesser values showed a slight improvement with Atabrine therapy. De Langen[16] observed that Atabrine and quinine somewhat diminished the regenerative powers of the blood system of previously barbitalized rabbits rendered acutely anemic by hemolysis or chronically anemic by blood letting.

In this study hematologic examinations (red and white cell counts, hemoglobin values, and differential counts) were made at weekly intervals on adult albino rats which were being given daily 5, 10, or 20 per cent of the acute oral LD_{60}. No significant effect of Atabrine on the red cell, white cell, or differential counts or on hemoglobin values was noted. However, the differential counts of the medicated animals deviated slightly from the normal as indicated by an increase in the polymorphonuclear and basophilic cells at the expense of the lymphocytes. The significance of this shift is open to question.

The Influence of Continued Ingestion of Atabrine on the Growth of Rats.—Wright and Lillie[6] observed the effects of progressively increased doses of Atabrine on the growth of rats weighing from 114 to 118 Gm. and examined the vital organs for histologic changes. They observed that large doses diminished the growth rate and resulted in more or less extensive histologic changes in the liver, spleen, and other organs, such as those described, but doses of 30 mg. per kilogram (approximately 5 per cent of the acute oral LD_{60}), given daily for one week, did not retard growth and the organic changes were either slight or absent. Because of the importance that Atabrine has assumed in replacing quinine in treating malaria, it appeared advisable to obtain additional data on this point.

Male rats weighing from 45 to 55 Gm. were maintained on a regular stock diet in individual false-bottomed wire cages. Atabrine was given six times weekly by stomach tube according to the dosage schedule shown in Fig. 5. On Saturday and Monday one-half again as large a dose was given to compensate for omitting a dose on Sunday. Groups A, B, and C each consisted of fifteen animals, whereas twenty were in Groups D and E. The doses given rats in Groups A, B, C, and D represented, respectively, 1, 2, 5, and 10 per cent of the LD_{60} for rats of this size. Inasmuch as it has been observed that rats weighing more than 100 Gm. are more sensitive to Atabrine, the daily dosage was reduced after the twenty-first day of the experiment, when the rats had attained an average weight of about 100 Gm., in order that the daily dose would maintain approximately the same relation to the acute LD_{60}. Twice weekly the animals were weighed and the dosage was adjusted to the new body weight. The growth curves are shown graphically in Fig. 5, in which an adverse effect on the growth of the animals in Groups C and D in comparison with that of those in Groups A, B, and E was already evident at the fourth day. At this point Groups C and D were divided and one-half of the rats in each group received 2 mol. of sodium bicarbonate for each molecule of Atabrine given thereafter.

Inspection of the growth curves indicates that the rats in Group A, receiving the lowest dosage of Atabrine, grew as well as or better than those in Group E, the controls, which grew less rapidly at first but maintained a steady rate of growth until the end of the experiment. The rats in Group B grew at a steady rate but by the end of the experiment appeared to be losing ground in comparison with the controls. The sodium bicarbonate supplements did not influence the toxicity of the Atabrine which was sufficient to retard the growth of the rats in Group C considerably and that of those in Group D markedly.

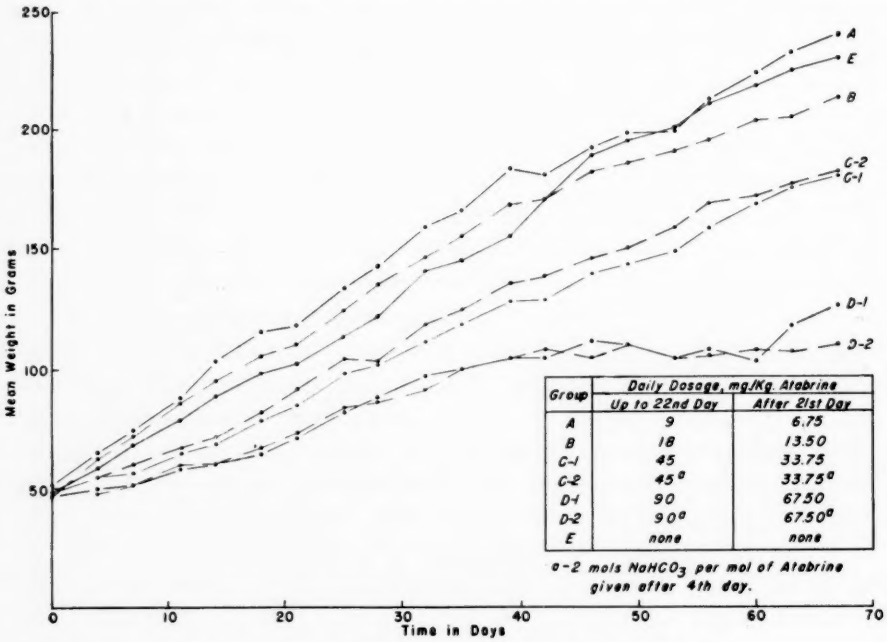


Fig. 5.—Influence of Atabrine on the growth of rats.

SUMMARY AND CONCLUSIONS

1. Data on the physicochemical characteristics of eight salts of Atabrine are presented.

2. When given by mouth to mice, the toxicity of quinine dihydrochloride, Atabrine base in water, Atabrine base in oil, and Atabrine dihydrochloride increases in the order named. The latter was only 25 per cent more toxic than quinine. The acute toxicity of four salts of Atabrine was determined in the mouse by three different routes of administration.

3. Atabrine manufactured by the American process from American intermediates was no more toxic to the rat than that manufactured from German intermediates by either the German or American process. The three specimens were deposited to the same extent in the tissues of dogs. While the effects of the three specimens on the growth of ducks were not different, the Atabrine from American intermediates produced significantly fewer deaths than those from German intermediates. Quinine sulfate, in doses comparable to those of Atabrine in chemotherapeutic efficacy, retarded growth to the same extent as Atabrine but did not cause any deaths.

4. Studies in which rats were given a massive dose of Atabrine by mouth show that high concentrations are reached in the tissues, and especially the liver, during the first eight hours and thereafter the amount of drug decreases, but measurable quantities still are present ten days after dosing. After forty-nine days of continuous administration of large doses, the livers of young rats showed a deposit of 4.58 mg. of Atabrine per gram of tissue and the blood level was 300 γ per 100 c.c. Detectable amounts were still present fifty-six days later. Pathologic examination showed that the injuries to the liver and other visceral organs resulting from large doses of Atabrine healed markedly during the two months after medication was discontinued.

5. The effect on the growth of rats of daily administration of Atabrine for seventy days was determined. At levels above 2 per cent of the acute LD_{60}, an adverse effect on growth was demonstrated. Sodium bicarbonate did not ameliorate the effect.

6. Continued administration of Atabrine had no significant effect on the blood of rats.

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EFFECT OF THE ORAL ADMINISTRATION OF THYMOL ON EXPERIMENTALLY INDUCED TUBERCULOSIS

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IN A previous paper, Searcy, McBurney, and Rowe[1] published a preliminary report of the results obtained with the clinical use of thymol as a therapeutic agent in six selected cases of pulmonary tuberculosis. During the course of its clinical trial, we were engaged in determining a like effect of this agent upon tuberculosis experimentally induced in guinea pigs. The results of these investigations are set forth herein.

The report of the clinical application of thymol on human beings preceded the findings on experimentally induced tuberculosis because opportunity for clinical trial was presented before completion of the latter, which even then gave evidence of prolonging the life of infected animals. Because of successful oral administration of thymol in the treatment of actinomycosis by Myers and Thienes,[2] Searcy and McBurney,[3] and Joyce[4] and the report by Barnes[5] of its use as an anthelmintic in Siam, with only two deaths among 82,000 treated individuals, no untoward results were anticipated.

In 1914, Perlman, Brown, and Raiziss[6] reported the use of thymol, menthol, and thymol-menthol combined as therapeutic agents against experimentally produced tuberculosis in guinea pigs infected with a virulent strain of bovine tubercle bacilli (Ravenel) by intramuscular injection of 0.4 c.c. of a 5 per cent solution in peanut oil.

Preliminary to our investigation, considerable work was entailed in the selection of the strain of organism, medium for growth, type of solvent for the thymol, and route of administration. A series of experiments, in which thymol in olive oil was administered intraperitoneally, gave rise to the formation of a profuse serous peritoneal exudate, resulting in the death of animals before evidence of tuberculous infection was established. This procedure was therefore discarded.

In all investigations the strain of *Mycobacterium tuberculosis* used was of human origin, isolated by us from the sputum of a patient with well-established pulmonary tuberculosis, and designated as strain J.* After many trials, Holmes'[7] medium was found best suited for growth, it being profuse and coarsely granular within three to four weeks.

Animals used were fully grown, healthy guinea pigs selected regardless of sex. Inoculations consisted of saline suspensions given subcutaneously in the inguinal region.

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Pathogenicity of the strain of *Mycobacterium tuberculosis*, using 1 mg., was determined simultaneously in eight animals; 75 and 100 per cent succumbed in six and eight weeks, respectively. Autopsy revealed gross and microscopic evidence of marked infection.

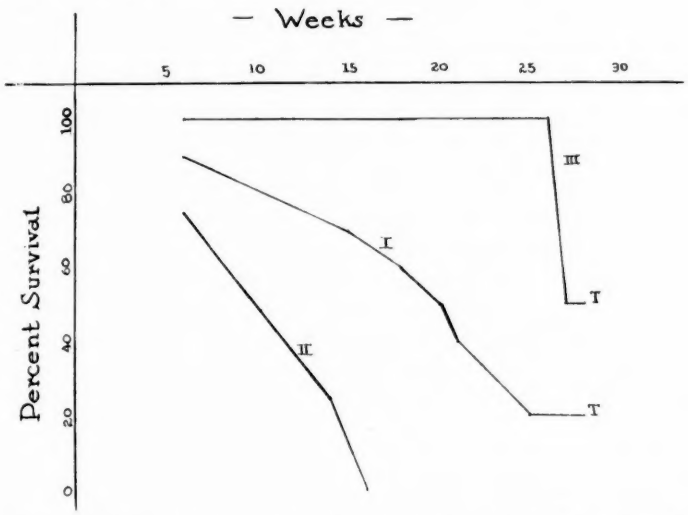
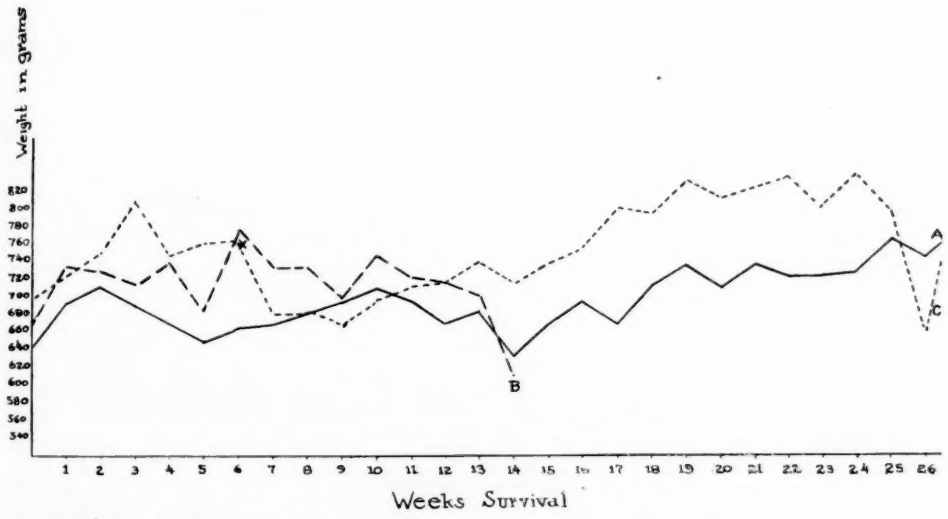


Fig. 1.—Percentage surviving at the number of weeks indicated following inoculation. *I*, Infected; thymol. *II*, Infected; no thymol. *III*, Thymol controls.



*One pig gave birth to two young.

Fig. 2.—Average weight curves of three groups of tested animals. *A*, Infected; thymol. *B*, Infected; untreated. *C*, Noninfected; thymol only.

Thymol medication experiments were also carried out on twelve animals receiving 1 mg. each of tubercle bacilli. Daily oral doses of thymol* in olive oil were given in amounts which corresponded to 10 gr. per 100 pounds of body weight. This was easily administered by means of a tuberculin syringe without needle. No therapeutic effect was observed.

*Merck's chemically pure.

An experiment using a larger dose of thymol and a smaller dose of organisms was next performed. Twenty animals were arranged in three groups, I, II, and III. Those in groups I and II were inoculated with 0.0005 mg. of culture J, the amount used by Feldman, Hinshaw, and Moses[8] in their study of the effect of promin (sodium salt of P,P′diamino-diphenyl-sulfone-N,N′-dextrose sulfonate) on experimental tuberculosis.

Animals in group III received no inoculation but were designated as a normal thymol control group. Thymol medication was given the pigs in this group simultaneously with those of group I; namely, one week following inoculation with tubercle bacilli. The amount of thymol used corresponded to 15 gr. per 100 pounds of body weight.

The animals in all three groups were distributed in large, well-aired, open cages as follows:

Group I (inoculated), 10 animals, thymol medication
Group II (inoculated), 5 animals, no thymol
Group III (normal thymol control), 5 animals, thymol only

All animals were well cared for, being given fresh water, oats, alfalfa hay, and fresh green vegetables daily. They were weighed one week prior to the experiment and at the end of each week during the experiment. Weight curves are shown in Fig. 2.

Results are shown in Table I and are expressed as the percentage surviving at the number of weeks indicated. This is likewise graphically portrayed in Fig. 1.

TABLE I

PER CENT SURVIVAL OF ANIMALS IN THREE GROUPS

WEEKS FOLLOWING INOCULATION	6	14	15	18	20	21	23	25	26	27	28
PER CENT SURVIVAL											
Group I Infected; thymol	90		70	60	50	40	30	20			20 (K)
Group II Infected; no thymol	75	25	0								
Group III Normal thymol controls; thymol only	*								100	50	50 (K)

*Death of one animal due to intestinal obstruction. Histologic sections showed chronic bronchitis and acute pneumonitis with no evidence of toxicity attributable to thymol.
(K), Killed.

GROSS PATHOLOGY

Autopsies were done on all animals. With two exceptions there was little difference in the gross appearance between the animals receiving thymol and those not receiving it. Generalized tuberculosis involving lungs, liver, spleen, mesentery, and inguinal lymph nodes was evident in the guinea pigs in groups I and II. Acid-fast stains made from lesions were positive.

Of the two exceptions, only a few small tubercules were seen in one area of the spleen in one instance and a single minute tuberculous abscess in the left inguinal region in the other. Interestingly, these two were treated animals surviving the length of the experiment and were killed at the end of the twenty-

eighth week. In view of gross findings noted in the animals in the inoculated groups, histologic sections were not made. In the pigs in group III, the thymol control group, no gross pathology was evident.

HISTOPATHOLOGY*

Histologic sections were made of the tissues of the four remaining animals in group III, who received thymol only, with the following findings: In the lungs of three there was slight active hyperemia; in one, slight edema and passive hyperemia. The liver of two showed moderate fatty changes; one, severe fatty changes; and one was normal. All showed a few phagocytes containing hemosiderin in the spleen. In the kidneys of two, there was slight active hyperemia; in one, slight tubular degeneration and hyperemia of the interstitial tissue; and in another, marked cloudy swelling of the tubules. Heart and suprarenals of all four were apparently normal.

With the exception of the one animal in this group that died during the sixth week of intestinal obstruction, whose lung section showed a chronic bronchitis and an acute focal pneumonitis, the remaining four pigs showed evidence of a moderate degree of damage evidently due to the toxic action of continuous and prolonged thymol medication.

WEIGHT CURVES

Curves *A*, *B*, and *C* in Fig. 2 show the average weekly weights of each group of animals in the experiment. The animals in each group gained weight the first few weeks. Since environmental conditions and diet were carefully controlled, the gain may be attributed to these factors. Following this initial gain the three curves present an interesting comparison. Curve *A*, representing infected animals receiving thymol, maintains a fairly constant or level curve. Curve *B*, representing infected animals untreated, shows a decline, whereas curve *C*, representing noninfected animals receiving thymol only, steadily rises. Fluctuations or drops in each curve occur prior to death, which was preceded by a loss in weight of the animal.

DISCUSSION

Only 25 per cent of the animals receiving no medication (group II) were living at the end of the fourteenth week and none the fifteenth.

Of the animals in group I, receiving organisms and medication, 90, 70, 60, 50, 40, 30, and 20 per cent were living at 6, 15, 18, 20, 21, 23, and 28 weeks, respectively, following inoculation. Twenty per cent of the treated infected animals survived twenty-eight weeks, which was 86.7 per cent longer than the untreated animals receiving similar doses of the organisms. The surviving animals may have lived longer but were killed at the end of the twenty-eighth week.

When all of the infected untreated animals had died from evident tuberculous lesions, 70 per cent of the infected animals receiving thymol were living and

*We wish to express our thanks to Dr. Jasper D. Bush and Dr. Mark C. Wheelock, Associate and Assistant Professor, respectively, Department of Bacteriology-Pathology, School of Medicine, University of Alabama, for the examination of and report on the histologic specimens, and Mrs. Irene Bush, of the Department of Pathology, for their preparation.

apparently well. At this time 100 per cent of the thymol control animals were likewise living and apparently well.

The average longevity of the animals in each of the three groups is given in Table II.

TABLE II

GROUP	DESCRIPTION	WEEKS	DAYS
I	Infected; thymol	18.7	(130.9)
II	Infected; untreated	11.6	(81.2)
III	Noninfected; thymol	27.0	(189.0)

Comparison of the figures in Table II reveals the following:

Infected animals receiving thymol lived 38 per cent longer than those receiving no thymol.

Noninfected animals receiving thymol lived 30.7 per cent longer than infected ones receiving thymol.

Noninfected animals receiving thymol lived 57 per cent longer than infected ones receiving no thymol.

Where we employed 0.0005 mg. of a human strain of the organism, Pearlman, Brown, and Raiziss[6] used one-tenth as much (0.00005 mg.) of a bovine strain. Three days following inoculation with the culture, they gave an initial dose of thymol intramuscularly. At five-day intervals, subsequent doses were given alternately in the right and left thighs. This was followed by a rest period of two weeks after the fourth injection. The average longevity of three groups of treated animals was ninety-two days, while that of a similar group of untreated ones was eighty-two days. The average longevity for the two methods is shown in Table III.

TABLE III

AVERAGE LONGEVITY (DAYS)

	MC BURNEY, CASON, AND SEARCY	PERLMAN, BROWN, AND RAIZISS
Infected; thymol	130.9	92
Infected; untreated	81.2	82

While the dosage of our human strain was ten times that of the bovine strain of Perlman, Brown, and Raiziss, apparently they were equally pathogenic for guinea pigs, if the time element alone is considered.

It is of interest to note that in comparing single dosage of thymol, both correspond, being equivalent to 0.3 gr. per kilogram of body weight. Considering the variation in frequency of medication and route of administration, the average life of infected animals receiving daily oral doses in our experiments was 42 per cent longer than those receiving alternate intramuscular injections at five-day intervals.

Had we used a dosage of our human strain corresponding to the bovine strain of Perlman, Brown, and Raiziss, it is not improbable that the average life of our treated animals have been much greater. This might well be the case if experimentally produced pulmonary lesions could be treated.

An interesting and striking fact is portrayed in the weight curves (Fig. 2) with respect to the animals in groups A (infected; receiving thymol) and C

(noninfected; receiving thymol only). Both show a parallel weight gain beginning the fourteenth week, at which time medication was given every other day instead of daily. This may indicate that while thymol medication was apparently responsible for the longevity of the guinea pigs in the infected group, an even greater interval between dosage might have been more effective. Such an hypothesis forms a basis for experiments now under investigation.

It is difficult to interpret the therapeutic effects of any drug used in experimental tuberculosis in terms of the pulmonary form on human beings, since so many uncontrollable factors enter the picture.

Experimental results obtained in guinea pigs represent the effect upon a widely disseminated infection, whereas it would appear that any hope for successful medication in human pulmonary tuberculosis depends upon treating early cases. The results we obtained with thymol medication in infected guinea pigs, wherein the average life of treated infected animals was nearly fifty days longer than that of the untreated ones, might be comparable to a marked span of years in man. While it is probable that oral thymol medication in early pulmonary tuberculosis, if of any therapeutic value, may not act specifically, it might result in arresting the disease to the extent that systemic treatment and natural body defenses would become more effective if used in conjunction with the time-honored methods employed by clinicians generally. This is mere conjecture and may be determined only by well-controlled clinical trial.

While it is true that some pathologic changes occurred in normal animals receiving thymol, it was mild in degree, the kidneys being more involved than other tissues. Apparently the toxicity evidenced by thymol in these experiments is far less than that manifested by certain sulfonamide compounds, especially when dosage is not adequately controlled.

Daily doses of 5 gr. of thymol, three times daily, given throughout a period of seventeen weeks to six patients with well-advanced pulmonary tuberculosis reported by Searcy, McBurney, and Rowe[1] exhibited no clinical manifestations of toxicity, and repeated urine examination failed to show evidence of kidney involvement.

Later clinical reports* on these patients showed that one died a year following treatment and another, four months following treatment from "far-advanced pulmonary tuberculosis." One was discharged home a year following treatment in "good condition" and has not been heard of since. Of the remaining three, fifteen months following treatment the disease was diagnosed "arrested" in two and "probably arrested" in the other. These cases were too few and the disease too far advanced to form a basis of conclusion. Hinshaw and Feldman[9] caution against hasty clinical evaluation of any therapeutic agent in human tuberculosis. They call attention to the tendency of many forms toward steady improvement without treatment. With equal emphasis they state that equal care must be taken lest some treatment be discarded prematurely.

*Information furnished through personal communication from Dr. Harry S. Rowe, Searcy State Hospital, Mt. Vernon, Ala., who treated these patients and who likewise submitted copies of reports of x-ray examinations and final diagnoses made by the Alabama State Department of Health, Division of Tuberculosis Control.

SUMMARY AND CONCLUSIONS

From the foregoing experiments it is evident that daily oral doses of 10 gr. of thymol per 100 pounds body weight produce no inhibitory effect upon massive doses (1 mg.) of virulent human strains of *Mycobacterium tuberculosis* in guinea pigs. However, where 0.0005 mg. of the organism is employed, 15 gr. of thymol per 100 pounds of body weight exert a marked inhibitory action as indicated by prolongation of the average life of animals so treated, nearly fifty days longer than those untreated.

When infected and noninfected animals were given similar oral doses of thymol on alternate days, their average weight gain was greater and more constant than when similar doses were given daily.

There was very little difference in the gross lesions, produced by the organisms, between the treated and untreated animals. However, fourteen weeks following inoculation, when all control untreated animals had died from generalized tuberculosis, 70 per cent of the treated animals were living and apparently well.

The daily oral dose employed, when given concurrently to a like group of normal guinea pigs, showed no observable evidence of toxicity throughout twenty-six weeks of treatment, since 100 per cent were living and apparently well at that time. Fifty per cent died during the twenty-seventh week, the remainder being killed at the end of the twenty-eighth week.

No gross pathologic changes were observed in post-mortem examination of the animals in this group, but histologic sections gave evidence of damage in a moderate degree.

It appears that thymol or thymol combinations administered orally as therapeutic agents in tuberculosis experimentally induced in animals and in early recognized human pulmonary forms where medication is adequately controlled are worthy of further investigation and clinical trial.

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PROGRESS

HUMAN COMPLEMENT

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BECAUSE of its unique and important role in natural immunity, and because of the practical application of its reactions to the laboratory diagnosis of disease, the humoral entity known as complement or alexin has received considerable attention from experimenters and clinicians. In the more than fifty years since its discovery, complement has been investigated by scores of workers in immunology and bacteriology, with the result that hundreds of papers have appeared in an attempt to establish a set of criteria for its adequate definition, to elucidate its constitution and the mechanisms of its various reactions, and to determine, with reference to actual physiologic conditions, its exact role in immunity.

From the functional viewpoint, complement has been defined as recently as 1939 as "comprising all of the activities of unheated serum."[1] This definition, to say the least, is too inclusive, because the thermolability of complement distinguishes it chiefly from the relatively thermostable antibodies. The functions of complement which have been established with some degree of certainty, excluding those ascribed to it on the basis of circumstantial evidence and coincidence, are concerned only with immunity and may be summarized broadly as follows: (1) the extracellular destruction of invading organisms or foreign cells when specific antibody is present (bactericidal action, bacteriolysis, hemolysis), and (2) together with specific antibody, the preparation of organisms or foreign substances so that these may be removed and destroyed by the phagocytic cells of the host (acceleration of opsonification). Complement may then be said to be an innate humoral factor which functions in cooperation with immune agents and with the cellular elements to destroy a variety of invading organisms and foreign substances.[2, 3]

It is to be noted that this definition omits the usual contrast with antibody, "complement is not increased in the course of immunization." The repetition of this statement has become habitual despite the fact that many workers have reported an increase in the complement of certain individuals suffering with infectious disease.[2] In this laboratory, too, such an increase has often been encountered, and until this phenomenon is better understood, it is suggested that the distinction be narrowed by saying that the augmentation of antibody in the course of immunization is more constant, of greater magnitude, and of different nature from that occurring with regard to complement.

From the Institute of Pathology, Western Reserve University, and the University Hospitals.
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From a constitutional standpoint, complement was long regarded as a "physicochemical state of fresh serum." The justification for this figurative description was the argument that complement is known only by its function, though a similar justification should then have existed for the classification of certain enzymes as "physicochemical states." It is now no longer necessary to cover ignorance in this fashion, for it is firmly established that complement consists of at least four serum components, three of which have been isolated in considerably purified form.

Until recently, knowledge of complement was confined almost wholly to guinea pig complement, and several extensive reviews of the subject have appeared.[1-5] The present review, therefore, is concerned mainly with a discussion of the constitution, immunologic functions, and variation in health and disease of the complement occurring in human serum. Of necessity much of this review is concerned with the work performed in this laboratory.

THE COMPLEX NATURE OF HUMAN COMPLEMENT

As in the case of guinea pig complement, human serum complement is composed of at least four components.[6] Originally each of these was defined by its participation in immune hemolysis, by a method for its inactivation with respect to this reaction, or by a method for its separation from the other components. Early attempts to "split" human complement by chemical means were inconclusive,[7, 8] but the recent isolation of serum fractions containing single component activities provides a material basis for the definition of at least three of the components.[9-11]

It is to be noted that the existence of at *least* four components is postulated. Since it is altogether possible that the present components may be further subdivided on the basis of activity, it is well not to be dogmatic in establishing limits for the complex nature of complement. Nevertheless, it is emphasized that the four components as now conceived are adequate for the constitutional definition of complement. All four components are necessary and sufficient for the production of immune hemolysis of sensitized sheep red blood cells as well as for the immune bactericidal reaction.[12]

In accordance with the terminology adopted for guinea pig complement and its components,[13] whole human complement is designated as human C,′ and the four components are designated as C′1, C′2, C′3, and C′4.[6]

C′1 is defined as that component which separates *completely* with the precipitate which forms when fresh human serum is dialyzed against phosphate buffer of pH 5.4 and ionic strength 0.02.[6] This precipitate, hereafter referred to as Fraction 1PP, also contains some of C′3 and C′4, but *no* C′2. C′2 is thus defined as that component which remains *completely* in the supernate (Fraction 1PS) after removal of the precipitate which forms in the dialysis described. Fractions 1PP and 1PS correspond functionally to the midpiece and endpiece, respectively, described for guinea pig complement.[2, 4]

That component which is completely and specifically inactivated or adsorbed by yeast or the insoluble carbohydrate of yeast, zymosan, is defined as C′3.[6, 14]

C′4 is defined as that component which is specifically inactivated by the addition of 0.20 c.c. of 0.16 M NH_4OH per cubic centimeter of human serum.[6]

In each of these cases, the serum or serum fraction lacking only one component is designated as a "specifically inactivated complement," indicated by the symbol siC′. Such specifically inactivated complements, used in appropriate amounts, are the analytic tools with which the investigator is able to determine the presence or absence of a given component in another serum or serum fraction. Since all four components are necessary for the production of immune hemolysis, a given serum which contains one of the four components will combine with that siC′ which *lacks* that component to complete the hemolytic system. The method in which siC′ is employed for the detection of the complement components is known as "specific reactivation."

In order to obtain more consistent results, we have undertaken the preparation of a standard complement and of standard specifically inactivated complements from the isolated complement components.

THE ASSAY OF COMPLEMENT AND ITS COMPONENTS

A given complement may be measured by its capacity for participation in any of the immunologic reactions with which it is associated, but the most consistent results are obtained with the almost universally employed method of immune hemolysis. The assay against sensitized sheep red cells has the additional advantage of being relatively simple to perform.

Many variations of the hemolytic titration have been proposed,[2, 15] the principal variables being time of incubation, dilution of complement, and amount of red cell substrate. However, the method most frequently employed is that in which increasing amounts of a given dilution of complement are allowed to act against constant amounts of substrate for thirty minutes at 37° C. Using this method, the titer of the complement may then be expressed as (a) the minimum amount of serum required to produce a beginning tinge of hemolysis (initial point titer),[16] (b) the amount of serum necessary to hemolyze exactly one-half of the standard unit of sensitized erythrocytes (50 per cent hemolysis titer),[17, 18] or (c) the minimum amount of serum required to hemolyze completely the standard unit of substrate (complete or 100 per cent hemolysis titer). The 50 per cent hemolysis titer, occurring in the range in which degree of hemolysis bears an almost linear relationship to the amount of complement, is probably the most accurate and reproducible expression of complement titer, although it is not always suitable for use in experimental work. In general, however, in a series of titrations it will be found that all three methods of expression of titer run parallel.

In 1936 Osborn[2] observed that the over-all titer of a given complement should be determined or limited by the titer of that component which is present in the least titer, just as the strength of a chain is limited by the strength of its weakest link. This conception was re-emphasized by Hegedüs and Greiner[19] and later by Heidelberger and Mayer,[20] the former in experiments determining the distribution of the components in the complements of various animal species and the latter in seeking to establish a correct basis on which to compare the titers of guinea pig and human complements.

On the basis of the hemolytic assay, Osborn[2] found the complement titers of various animal sera to fit the following scheme:

$$\text{Guinea pig} > \text{human} = \text{rat} > \text{rabbit} = \text{cat}$$

Da Costa Cruz and Penna[21] found that human, monkey, and dog complements were of the same order of activity. We have estimated that guinea pig serum contains about five to six times the effective complement contained in human serum and that human serum is about two to three times more active than rabbit serum.

Hegedüs and Greiner[19] demonstrated that midpiece (C′1) was the component of least titer, and therefore the limiting component, of guinea pig complement. They also postulated that endpiece (C′2) was the limiting component of human complement, while it has been shown in this laboratory that C′3 is present in an effective titer equal to, or less than, that of C′2.[22]

If a normal human complement is treated with the various testing complements (siC′) at the level of *initial* hemolysis, it is found that the effective concentrations of the components as determined by the reactivation of the siC′ for the hemolytic system range as follows:

$$C'1 \geqslant C'4 > C'2 \leqslant C'3.$$

The knowledge, then, of the effective concentration of a given component must be considered in any study of complementary activity.[22]

THE FIRST COMPONENT (C′1)

C′1 of human complement has been prepared by fractional precipitation of human serum with ammonium sulfate at 1.4 M, extraction of the resulting precipitate with phosphate buffer of pH 5.4 and ionic strength 0.02, re-precipitation with ammonium sulfate between 1.0 and 1.8 M, and final re-extraction of this precipitate with the phosphate buffer.[9] Obtained in this manner, C′1 has been characterized as a euglobulin with an electrophoretic mobility of 2.9×10^{-5} in veronal buffer of pH 7.8 and ionic strength 0.1. Examined in the Svedberg oil-turbine ultracentrifuge, the C′1 preparation was shown to contain a major component which had a sedimentation constant of 6.9 Svedbergs. C′1, isolated by the methods described, constitutes from 0.6 to 0.8 per cent of the total serum protein. With the aid of Lustig and Kondritzer,[23] an elementary and amino acid analysis of C′1 has been obtained; these results are considered in another section of this review together with those obtained in a similar analysis of C′2.

Purified C′1 is best preserved in 4.5 per cent NaCl, or in solutions buffered to pH 6.6 and containing greater than 0.9 per cent NaCl. So dissolved, C′1 may be kept unchanged in the frozen state for at least two years. It withstands the lyophilization process and accordingly may be preserved by drying from the frozen state.

Recently, Cohn and co-workers[24] have reported the concentration of human C′1 in their Fraction III-2.

Immunologically, purified C′1 is inactive per se in both the hemolytic and bactericidal systems, but when combined with the other three components, it is fully active against sensitized sheep erythrocytes and sensitized *Vibrio comma.*[10]

THE SECOND COMPONENT (C′2)

Two factors have militated against the purification of C′2. In the first place, this component is a minimum component in human complement and therefore the yields tend to be poor; and in the second place, human C′2 precipitates at a much higher range of ammonium sulfate concentration than does guinea pig C′2, thus making its separation from albumin much more difficult. Nevertheless, a highly purified fraction was prepared by precipitation of human serum at pH 6.8 between 2.4 and 2.6 M ammonium sulfate.[10] While all preparations of C′2 obtained by this method contained about 50 per cent albumin as determined electrophoretically, they also contained a globulin with an electrophoretic mobility of 2.6×10^{-5} in veronal buffer of pH 7.8 and ionic strength of 0.1.[25] It should be remarked that guinea pig C′2 was found to be an alpha globulin containing over 10 per cent carbohydrate and having almost full C′4 activity.[26] The human preparation, in contrast, had about 3 per cent carbohydrate and no C′4 activity, and if it contained any alpha globulin, this latter fraction was masked by combination with another serum protein.

Again with the aid of Lustig and Kondritzer[23] an elementary and amino acid analysis of C′2 was secured. In comparison with the similar analysis of C′1, the results show that C′1 has a higher concentration of hydroxy amino acids (serine, threonine), tyrosine, and tryptophane. C′2, on the other hand, has a higher content of sulfur-containing amino acids (cystine and methionine) and the basic amino acids (arginine and histidine). The higher carbohydrate content of C′1 finds its expression in higher carbon, nitrogen, and humin-nitrogen values. It should be recalled that the C′2 preparation contains about 50 per cent albumin, and this is probably reflected in the higher cystine value.

In their recent studies on the fractionation of human plasma, Cohn and his associates[24] concentrated C′2 in their Fraction IV.

When dissolved in 0.9 per cent NaCl and buffered to pH 6.6,[27] C′2 may be preserved in the frozen state for at least two years. It may also be preserved by drying from the frozen state.

Purified C′2 per se is not active in immune hemolytic or bactericidal systems, but is fully active when combined with the other three components.[10]

If human serum is heated at 50 to 52° C. for thirty minutes, its over-all complementary activity is lost, and simultaneously C′2 disappears.[6] However, it is not to be inferred necessarily that C′2 is more thermolabile than C′1, for it is more probable that the heat inactivation is a function of concentration, and since C′2 is present in lesser concentration than the other protein component (C′1), its activity disappears first.[22]

THE THIRD COMPONENT (C′3)

This component has not been isolated from either guinea pig or human serum in any great degree of concentration or purification. Refractionation of Fraction 1PP (see above) at −10° C. with 30 per cent ethanol results in a precipitate

containing 70 per cent C′3 and small amounts of C′1 and C′4.[28] However, much remains to be done before it can be said that a purified C′3 is available, and work in this direction is in progress.

When human serum is treated with zymosan and then examined in the Tiselius apparatus, a disturbance in the gamma globulins is noted.[29] This may be indicative of an association of C′3 with the gamma globulins, as is the presence of most of the serum C′3 in Fraction 1PP. However, it should be remembered that the "specificity" of the zymosan for C′3 is only relative, and that often small amounts of C′1, C′2, and other proteins are adsorbed as well, and this may very well account for the observed disturbance in the Tiselius diagram.

Thus, outside of the fact that it is a minimum component in human complement,[22] that it is relatively unstable,[27] and that there are indications that it acts as an enzyme,[2, 30] very little is known of the chemical nature or properties of C′3.

THE FOURTH COMPONENT (C′4)

The fourth component, originally defined by its specific inactivation by dilute ammonia and other amino compounds,[31] has been postulated to be a reactive carbonyl group attached to a protein carrier.[32] In guinea pig complement, its persistent association with C′2 through all stages of purification inspired the conception that C′4 was a prosthetic group attached to C′2. However, in the case of human complement, C′4 has been found associated with fractions containing C′1 as well as those containing C′2[6] and it was not present in either purified C′1 or C′2. It must be noted, however, that C′4 associated with C′1 in Fraction 1PP was found to be very labile, whereas in association with C′2 in Fraction 1PS it was relatively stable.[27] The failure to find the fourth component in the purified human C′2 preparation may have been due to the destruction of C′4 in the course of the many ammonium sulfate precipitations.

Human C′4, obtained relatively free from the other components, was prepared by dialysis of Fraction 1PP (see above) against a phosphate buffer of pH 6.6 and ionic strength 0.04. The supernate which was thereby obtained constituted from 1 to 2 per cent of the total serum protein and contained 90 per cent C′4 activity in one unit along with small amounts of C′1 and C′3. These latter two components were removed by treatment with an excess of zymosan. Prepared in this manner, C′4 is best preserved by solution in phosphate buffer of pH 6.6 made isotonic with NaCl.[33] Like C′1 and C′2, C′4 may be preserved by drying from the frozen state.

The purified C′4 preparation is extremely sensitive to dilute alkali and amino compounds such as ammonia and hydrazine, inactivation occurring within one minute at room temperature.

HUMAN COMPLEMENT IN BACTERICIDAL PHENOMENA

It has been shown[2, 34-36] that the bactericidal and bacteriolytic effects of blood serum are dependent upon the interaction of two serum constituents, the thermolabile complement and the thermostable antibody. Recent studies[12] with human serum have confirmed the older findings on the functions of complement and antibody in these phenomena and, especially on the basis of the recent

knowledge of the constitution of complement, have provided new information on the particular role of human complement in the destruction of organisms. The results of these studies are summarized below.

Fresh adult human serum was shown to exert marked bactericidal and lytic action against a strain of *Vibrio comma*. Heated adult serum and fresh infant serum of normal complement titer did not exhibit these properties. However, a combination of these two sera in the proper proportions was almost as bactericidal as fresh adult serum. Fresh infant serum could also be rendered bactericidal by the addition of inactivated specific rabbit antiserum which in itself was likewise nonbactericidal. By inference, then, adult serum must be bactericidal by virtue of its complement and naturally occurring antibody. Also, by measuring the bactericidal effects of fresh infant serum and specific antiserum combined in varying proportions, the degree of bactericidal action was found to be dependent on the amount of complement as well as on the amount of antibody.

In the same study[12] it was further observed that specific inactivation of any one of the four components of human complement destroyed the bactericidal powers of the treated adult serum. Combination of specifically inactivated complements resulted in the restoration of the bactericidal properties almost completely. All four complement components are therefore necessary for bactericidal and bacteriolytic effects. It was also noted that the bactericidal power of normal adult serum could be fortified by the addition of an excess of any of the specifically inactivated complements, the siC′ lacking C′1 but containing C′2 and some C′3 and C′4 (Fraction 1PS) being particularly effective. The fortification of the bactericidal action of complement by the addition of C′2 and C′3 is supportive evidence that these two are the components of minimum titer in human complement.

Hemolytic tests run in parallel with the bactericidal experiments indicated a qualitative relationship between these two phenomena.

THE FIXATION OF HUMAN COMPLEMENT BY BACTERIA

Aside from the accepted fact that a suspension of bacteria can fix human complement in vitro, little was known of the nature of this fixation. On the basis of the newer knowledge of the structure of complement, a study was made of the nature of complement fixation by six species of bacteria including both coccal and rod forms.[37]

Fixations were performed with unsensitized bacteria and with the same organisms sensitized by specific rabbit antisera. The effects on the complement were determined by the method of specific reactivation after fixation and separation of the organisms. From this study the following conclusions were drawn.

If the quantity of bacteria employed was sufficiently large, or the incubation period sufficiently prolonged, there was a complete loss of hemolytic activity of the complement. Since complete removal or inactivation of at least one component is necessary for hemolytic inactivation, at least one component must then have been entirely fixed to account for this effect. In agreement with this, at the initial point of removal of over-all hemolytic activity, C′2 or C′3 was found to have been removed completely, and there was a partial decrease in the ac-

tivities of the other components. One exception was noted in the case of *Streptococcus viridans*, in which fixation of 50 per cent of C′2 and C′3 resulted in the complete inactivation of the complement. It is worthy of note that C′2 and C′3 are also the components of minimum titer in human complement.

Further, except for a slight increase in the *rate* of fixation, no other difference was observed between the effect of sensitized and unsensitized bacteria on the complement. As was pointed out, the presence of natural or group-related antibodies in the sera would tend to obscure expected differences in the nature of fixation in the presence or absence of antibody.

Although definite differences existed in the manner of fixation by various organisms, it was not possible to correlate the mechanisms with any characters of the bacteria themselves. Likewise, definite conclusions regarding the specificity of the fixation cannot be made at this time, although the differences in fixation exhibited by various species of organisms would indicate some degree of specificity. Moreover, one species of organisms, *Neisseria intracellularis*, when employed without antiserum, fixed only a slight amount of C′2 and no other component even after many hours of incubation. Sensitized organisms of this strain fixed all of C′2 after several hours, and a portion of C′4 could be removed on prolonged fixation beyond this time. This particular strain of *Neisseria intracellularis* was resistant to the bactericidal action of several different fresh normal adult sera, which indicated a lack of specific antibody in the sera.

The assay of complement remaining after bacterial fixation revealed which components were fixed to the organisms, but it did not disclose the fate or function of the fixed components. However, in experiments with *Vibrio comma*, it was found that when those components not fixed were added in sufficient amount to the washed treated organisms, bactericidal action resulted. Thus components removed from complement by bacteria were not destroyed but retained their property of participating in subsequent complement reactions. It was further noted that the hemolytic functions of the complement components remaining in the sera after fixation by bacteria closely paralleled the bactericidal functions of the fixed components.

We are convinced that future studies on the mechanism of fixation must take into account the effective concentrations of the individual complement components as suggested in another paper.[22]

In a study of the quantitative aspects of fixation of human complement to specific immune precipitates of egg albumin, the specific carbohydrate of the Type III pneumococcus (S III), and S II, Heidelberger and Mayer[20] concluded that from 0.03 to 0.05 mg. of C′ nitrogen per milliliter of human serum adds to these precipitates.

THE OPSONIC ACTION OF HUMAN COMPLEMENT

In 1941, before the human complement system had been adequately investigated, Ecker, Pillemer, and Kuehn[38] studied the opsonic action of normal human serum on a strain of *Staphylococcus aureus*. No relationship was found to exist between the hemolytic titer of human serum and its antistaphylococcic opsonic powers. Heat inactivation readily and markedly reduced the normal human serum opsonins. Ammonia, used in what is now known to be approximately

optimum amounts for the inactivation of human C′4, caused reduction of opsonic power; adsorption of the serum with zymin (a yeast preparation) did not change the opsonic capacity. Separation of human serum by carbon dioxide-saturated water into a precipitate (corresponding roughly to Fraction 1PP above) and a supernate (roughly Fraction 1PS) resulted in a division of the opsonic power between the two fractions, the major portion residing in the supernate.

In the past, Gordon and Thompson,[39] Elvidge,[40] and Welch, Brewer, and Hunter[41] have presented evidence to show the mutual independence of opsonin and complement. Ward and Enders[42] summarized the matter by observing that human complement does not produce an absolute increase in the promotion of phagocytosis by the serum, but, acting probably as a biocatalyst, accelerates the rate of phagocytosis.

While it is already obvious that the *total* complement complex is not required for the acceleration of opsonification, experiments now in progress with purified components will determine which components are of importance in the opsonic reaction.

THE VARIATIONS OF COMPLEMENT IN INFECTIOUS DISEASE

In 1902 Wassermann[43] proposed the view that the serum complement concentration could be employed as an index of general resistance. Cadham[44] thought that a high complement titer was beneficial in immunity. That low general immunity may be coincident with low complement titer was shown in the case of the complement-deficient guinea pigs bred by Moore[45] in 1919. The sera of these animals were deficient in C′3[46, 47] and perhaps C′4, and the animals themselves were highly susceptible to natural infections and to the injection of pathogenic bacteria.

In general, the complement of an individual is very close to, and constant with, the titer found to be average for the species. This statement applies only to the complement of healthy individuals, however, for the literature lists many variations in disease. In 1912 Dick[48] found that in pneumonia the complement is low before crisis and high the second to third day following crisis. Recently, Rutstein and Walker[49] reported that complement diminishes during the course of pneumonia and particularly following the administration of an antipneumococcal serum. These authors also reported a drop in over-all complement titer in six of eleven cases of serum disease. Thomas and Dingle[50] observed that the intravenous injection of rabbits with a concentrated rabbit antimeningococcal serum resulted in the disappearance of hemolytic complement for as long as twenty-four hours. Interesting clinical responses have also been reported in cases in which fresh serum, containing complement, was used together with the specific sera in meningococcal and influenzal infections.[51-53]

In 1929 da Costa Cruz[54] reported a study of 103 cases of yellow fever, in 94 per cent of which he had observed a markedly diminished complement which rapidly improved in titer during convalescence. It should, however, be emphasized that the liver is diseased in yellow fever, and the low complement may in some measure be related to the interference with production of the protein components of complement.

The literature on complement in tuberculosis is contradictory, as is that on syphilis. In both trypanosomiasis and malaria decreased complement titers have been noted.[1, 2]

With the view of resolving, if possible, the many contradictory reports on variations of complement in infectious disease and determining the mechanisms underlying these variations, we have undertaken a long range and serial study of a variety of cases. In this review only a few tentative conclusions, obtained in a study of approximately 300 cases, are reported as follows:

1. While over-all complement titer often changes in the course of infectious disease, the extent and direction of the changes are by no means always similar in different diseases, nor even in individual instances of the same disease.

2. In fourteen cases the over-all complement fell to zero or near zero, usually coinciding with a serious phase of the patient's condition. With improvement of this condition the complement titer would tend to return to the normal value. However, of these fourteen patients, approximately one-third died, whereas the fatalities of all cases amounted to less than 5 per cent.

3. In almost all of those patients showing a decrease in over-all complement titer there was a striking decrease or disappearance of the *fourth component (C′4)* and a decrease in the second component (C′2). For instance, all of those showing zero complement had no C′4, a fact made more striking if it is recalled that this component is a component of maximum titer in normal complement. C′1 varied only slightly, while C′3 did not vary significantly. When diminished titers improved, there was a concomitant improvement in C′4 and C′2 titers.

4. There appears to be some prognostic value in the complement titration in those patients in whom complement titer decreases, and in particular in following C′4 activity. However, too few cases have been studied to permit this statement to be made with certainty.

5. In numerous cases of scarlet fever, measles, and erysipelas, the patients showed *increased* complement titers, all four components apparently participating in this change.

A detailed analysis of these cases will be published elsewhere, and the relationships of complement titer to serum protein concentration, white blood counts, and temperature will be considered. Future work will be concerned with the relationship of in vivo complement fixation, if this is shown to occur, to the lowering of complement titer and with a study of complement variation in the sera of rabbits with experimentally produced infections. Eventually, treatment of infected rabbits will be attempted with purified or concentrated complement preparations.

SUMMARY

A constitutional basis for human complement, similar to, though not identical with, that previously established for guinea pig complement, has been presented. Human complement is composed of at least four components, each of which is described by a method of specific inactivation or separation. Three of these have been prepared from human serum by various fractionation procedures, and only the third component (C′3) remains to be purified adequately. It is established that the second and third components (C′2 and C′3) are the

components of lowest titer in human complement and thus determine the over-all titer of the serum.

All four components are required for the immune hemolytic and bactericidal reactions, but the exact role of the components in the acceleration of opsonification is as yet unknown. Six species of bacteria, including coccal and bacillary forms, have been shown to inactivate human complement, principally by the fixation of the second or third components. The fixed complement components retain their capacity for participation in subsequent immunologic reactions such as bactericidal action.

Complement varies in infectious disease, and in those patients in whom it drops in titer to zero or near zero, the prognosis is grave. When the complement disappears in these, it is by virtue principally of the inactivation, fixation, or inhibition of the fourth component (C′4) and secondarily of the second component (C′2).

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LABORATORY METHODS

TUBULAR RESORPTION TEST

With Results Obtained in Nineteen Cases of Acute Nephritis

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HYPOPHYSIS posterior lobe extracts have been used repeatedly for estimating renal function.[1-14] The various methods consist essentially in determining the specific gravity of urine following the administration of a quantity of extract; the conclusion has been reached that when the kidney loses its capacity to concentrate urine, the antidiuretic hormone also loses its effect. Since 1940[15] we have been using pitressin to test the functional capacity of the kidney, but, instead of taking into account urine concentration, we have made direct measurements of the antidiuretic effect obtained after standardized administration of water and pitressin.

This paper gives a simplification of the technique originally used and the results obtained in nineteen patients with acute nephritis.

Procedure.—The method is as follows:

1. The patient is kept fasting and lying in bed.
2. One liter of water in ten minutes is given.
3. Five units of pitressin are injected intramuscularly.
4. A catheter is introduced into the bladder and two urine specimens collected at thirty and ninety minutes, respectively.
5. The first specimen is discarded; the second is measured and the figure obtained is the result of the test.

The maximum antidiuretic effect is obtained between thirty and ninety minutes after the pitressin injection.

Results in Normal Subjects.—This tubular resorption test was carried out in forty normal male individuals between the ages of 20 and 25 years.[15] The results obtained varied between 16 and 76 c.c. of urine, with an average of 36.2 c.c. We have never seen normal subjects with values higher than 80 c.c., and, therefore, we consider as abnormal any values above this figure.

The test is well tolerated; there is obvious vasoconstriction of the skin and a rise of from 20 to 30 mm. in blood pressure, not lasting more than 15 to 20 minutes after the pitressin injection.

Results in Acute Nephritis.—The test was carried out in nineteen cases of acute nephritis occurring in men between the ages of 19 to 21 years. These patients presented the typical picture of acute nephritis, the important symptoms and evolution of which are detailed in Table I. They were admitted to

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TABLE I

TUBULAR RESORPTION TEST IN ACUTE NEPHRITIS

CASE	DAY FROM THE ONSET OF DISEASE	T.R.T.	U.C.	BLOOD PRESSURE	HEMATURIA	ALBUMINURIA	URINARY CASTS	EDEMA	BLOOD UREA (MG. %)	SED. RATE
1	9	68	Mx 75	150/ 90	+	+	0	+	26	112
	35	62		110/ 64	0	0	0	0		18
	42	–								44
	49	*112*		118/ 72	0	0	0	0		
	85	87		125/ 75	0	0	0	0		2
2	9	*210*	Mx 100	168/ 70	++	+	+	+	54	18
	16	*154*	Mx 66	140/ 70	+	+	0	0	45	18
	30	64		130/ 80	+	0	0	0		15
	44	67		126/ 80	0	0	0	0		5
	58	*84*		130/ 78	0	0	0	0		
	77	47		130/ 85	+	+	0	0		5
3	15	79	Mx 65	158/ 85	+	0.2 Gm. %	+	+	63	75
	24	*111*	Mx 125	124/ 62	+	+	+	0	35	20
	37	40		110/ 70	0	0	0	0	48	5
4	8	71		160/100	++			+	50	10
	16	*134*		150/ 90	+	0	0	+	40	10
	26	70		128/ 70	0	0	0	0	40	10
	45	*129*	Mx 64	136/ 68	+	0	0	0		2
	53	*84*		124/ 70				0		5
5	11	*158*		190/110	+	0.25 Gm. %	+	+	50	10
	20	*93*	Mx 93	130/ 90	0	0	0	0	30	37
	35	*82*		120/ 70	0	0	0	0		27
	53	65		120/ 70	0	0	0	0		2
6	27	*107*	Mx 100	136/ 80	+	+	+	0	64	32
	38	79	Mx 60	130/ 90	+	+	+	0	34	3
	48	60		130/ 80	+	+	+	0		9
7	25	*220*	Mx 50	170/110	+	0.10 Gm. %	+	+	60	50
	36	*165*		158/108	0	+	0	0	40	20
	52	*86*	Mx 133	138/ 96	+	+	+	0	44	20
	66	64		130/ 80				0		10
8	29	60		110/ 70	+	0	0	+	55	10
	44	*118*		108/ 60	+	+	+	0		2
	55	*154*		110/ 70	0	0	0	0	35	2
	72	79	Mx 71	126/ 74	0	0	0	0		
9	5	–		178/120	+	+	+	+	64	20
	19	*130*		150/120	0	0	0	0	27	5
	31	66		145/110	0	0	0	0		5
	42	65	Mx 90	132/ 92	0	0	0	0		5
	62	56		124/ 86	0	0	0	0		2
10	5	–	Mx 78	240/140	+	+	+	+		21
	17	35	Mx 89	170/105	+	+	0	+	56	8
	32	46		166/ 65	0	0	0	0	20	4
	48	30		144/ 75	0	+	0	0		
11	20	–	St 32	210/120	+	0.20 Gm. %	+	+	45	42
	33	75	Mx 33	150/ 90	+	0.10 Gm. %	0	0	70	46
	47	*145*		122/ 82	+	+	0	0		72
	68	66	Mx 66	142/ 84	+	+	0	0		35
12	3	–	Mx 100	190/140	+	0.20 Gm. %	0	0	56	
	16	78		150/110	+	0.02 Gm. %	0	0		
	34	*81*		155/110	0	+	0	0	55	
	48	71		156/108	0	+	0	0	55	
	91	74		160/110	+	+	+	0		
13	19	–		145/ 85	+	+	0	0	42	20
	29	*105*		136/ 70	+	+	0	0		25
	43	70		140/ 90	0	0	0	0		5
	57	87		150/ 86	++	+	+	0		10
	80	*92*		142/ 90	+	0.01 Gm. %	0	0	49	2
	100	79		134/ 90	+	+	+	0		2
	110*	–		130/ 84	+++	+	+	0		25
	122	*86*		133/ 82	+	+	0	0		10

*Tonsillectomy.

†Dental extraction; apical granuloma.

TABLE I—CONT'D

CASE	DAY FROM THE ONSET OF DISEASE	T.R.T.	U.C.	BLOOD PRESSURE	HEMATURIA	ALBUMINURIA	URINARY CASTS	EDEMA	BLOOD UREA (MG. %)	SED. RATE
14	22	*138*		160/110	+	0.05 Gm. %	0	+	55	25
	36	*167*	Mx 52	134/ 80	+	+	+	+	64	40
	53	*180*	Mx 70	124/ 80	+	+	+	0		90
	66	*145*		124/ 75	+	+	0	0	50	90
	82	*127*		130/ 80	+	+	0	0	50	40
	102	*107*	Mx 72	128/ 82	+	+	0	0		30
	113	*88*		132/ 85	+	+	+	0		10
	129	72	Mx 129	125/ 85	+	+	+	0		10
15	13	*142*	Mx 63	170/ 95	++	0.15 Gm. %	+	+	75	54
	27	*116*	Mx 47	130/ 80	++	0.20 Gm. %	0	0	30	62
	41	*159*		130/ 70	+	+	0	0		49
	67	*99*		128/ 60	+	+	+		40	23
	82	*113*	Mx 81	128/ 60				0		20
	91	*97*		118/ 60	+	0	0	0	45	16
	109	78	Mx 152	126/ 62						5
	123	*84*		120/ 64						2
16	11	–	St 29	160/130	+	0.05 Gm. %	+	+	145	40
	34	*85*	Mx 85	130/100	+	+	0	0	17	20
	41	40		120/ 80				0	22	20
	54	79		120/ 78	0	0	0	0		6
	93	63		120/ 80	0	0	0	0		3
17	17	–		150/ 60	+	0.05 Gm. %	0	0	30	70
	34	*90*		130/ 70	+	0	+	0	36	38
	43	*94*		132/ 60	+	0	0	0		30
	56	*95*		120/ 75	0	0	0	0		19
	85	61	Mx 100	122/ 70	0	0	0	0		8
	108	49		120/ 70	0	0	0	0		
18	9	*120*	Mx 93	180/100	++	0.025 Gm. %	+	+	60	85
	16	75	St. 61	130/100	0	+	0	0	35	65
	30	79		132/ 80	0	0	0	0		10
	44	62		140/ 95	0	0	0	0		2
	58	59		132/ 88	0	0	0	0		
	60†	–								
	65	34		134/ 82	+	+	0	0		5
	101*	–		120/ 80	+	+	+	0		42
	106	45		120/ 80	+	+	+	0		25
19	4	–	St 93	155/ 70	+	0.025 Gm. %	+	+	150	70
	14	*134*	Mx 42	142/ 76	++	0.025 Gm. %	+	0	90	90
	50	56	Mx 170	142/ 66	0	0	0	0	42	85
	70*	–		130/ 60	++	0	0	0		
	105	50		120/ 62	0	0	0	0		26

the hospital early in the course of the disease and remained for periods which varied between 45 and 135 days. The treatment consisted of rest, diet, and general care only.

The test was made at an early stage and repeated periodically throughout the patient's stay in the hospital. The following determinations were made simultaneously: urine analysis, blood urea, sedimentation rate, blood pressure, and, occasionally, urea clearance test. An average of five tests was performed on each patient, the results of which are detailed in Table I. They were as well tolerated as in normal subjects.

Only one patient gave a normal value; abnormal figures, for example, higher than 80 c.c., were obtained in eighteen patients. The antidiuretic action of pitressin was decreased in all patients and had even disappeared completely in some of them; that is, after the ingestion of a liter of water the diuresis was the same with or without pitressin.[16] The results obtained in the first stages of

the disease were, in general, the most abnormal, gradually decreasing to normal values toward the later stages.

In fourteen of the eighteen patients it was possible to repeat the test until the normal level had been reached. This occurred between 30 and 135 days after the appearance of the disease. In ten of these patients all other symptoms of the disease had disappeared by that time, while in four there were still signs of hematuria, albuminuria, and urinary casts.

In nine of the eighteen patients, the last abnormal test, when recorded, was the only remaining sign of the disease; in six of these it eventually returned to normal. On the other hand, in the remaining nine the last abnormal test was still accompanied by hematuria, albuminuria, urinary casts, or increased sedimentation rate.

In the patients in whom it was possible to carry out urea clearance tests, the results obtained, with very few exceptions, returned to normal much before the tubular resorption test. The blood pressure always went back to normal much before the tubular resorption test.

DISCUSSION

Pitressin acts directly on the renal tubules, causing an increase in water resorption and consequently a decrease in diuresis.[17, 18] This antidiuretic effect is decreased or disappears completely in dogs intoxicated with uranium salts, as demonstrated by Molitor and Pick[19] and Hayman, Shumway, Dumke, and Miller.[20] As is well known, the uranium salts selectively affect the renal tubules. It is most probable that when the renal tubules are damaged in man, whatever the cause, the antidiuretic effect is also diminished or even disappears and that the extent of the diminution is proportional to the extent of the tubular lesions.

As water resorption can undoubtedly be considered one of the principal functions of renal tubules, we find that in estimating the loss of that function, a direct measurement of urine volume seems more appropriate than does the indirect method of measuring urine concentration. Moreover, we consider that determining urine volume instead of urine concentration has the following advantages: (1) urine volume is independent of salt and protein content of the diet taken during the previous days; (2) it eliminates the necessity of giving a test meal; (3) it is less affected by the presence of edema. From a practical standpoint the whole test lasts only one and one-half hours and is well tolerated even in the early stages of nephritis.

Results obtained in acute nephritis lead us to presume that tubular function is affected at a very early stage[16, 21]; moreover, the test tends to normalize itself very late, generally after all other signs of the disease have disappeared, indicating that in many cases tubular function is the last to return to normal. As only one of the nineteen patients gave normal values for the tubular resorption test in spite of the presence of the other symptoms, it might be that the disease in this case was of a different kind and that consequently there might exist two types of acute nephritis.

SUMMARY

A tubular resorption test using the antidiuretic hormone of the posterior pituitary has been proposed for estimating renal tubular function. The tech-

nique consists of the following procedures: after the ingestion of 1 liter of water and the injection of 5 units of pitressin, urine is collected during the interval between thirty and ninety minutes after the water intake. In normal subjects the diuresis during that period was from 16 to 76 c.c. (average, 36.2); a diuresis of more than 80 c.c. is considered abnormal.

Of nineteen patients with acute nephritis, eighteen gave abnormal values during the course of the disease with, in general, a decrease toward normal values in the later stages. In most of the patients the tubular resorption test was one of the last signs to return to normal.

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A SYNTHETIC MEDIUM FOR DETERMINING CITRATE-UTILIZING, HYDROGEN SULFIDE-FORMING BACTERIA

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KOSER,[1] working with the salts of certain organic acids, demonstrated that the genus Aerobacter can be distinguished from *Escherichia coli* through the ability of the former to utilize citric acid as a sole source of carbon, whereas *Esch. coli* cannot. The ability to utilize citric acid as a sole source of carbon is shared, also, by *Escherichia freundii* and other intermediates of the coli-aerogenes groups as well as by most species of the Salmonella and by many of the "paracolon" group of organisms.

In 1926, Simmons[2] adapted Koser's findings into his citrate agar medium. *Esch. freundii,* most of the genus Salmonella, and the "true" paracolons, which cannot be distinguished from the genus Aerobacter on either the Koser or the Simmons media, may be separated from it by their ability to form hydrogen sulfide on peptone media.

It occurred to me that possibly the value of these citrate media might be enhanced if a suitable hydrogen sulfide indicator could be incorporated into one or both of them in order to differentiate further those organisms capable of utilizing citric acid as a sole source of carbon. If this were found to be possible, it might eliminate the necessity of using two different media for this purpose.

Since Koser[1] noted that "identical results could be secured with either potassium, ammonium, or sodium citrate," there was the possibility that ferric ammonium citrate might be used as the source of both citrate and iron. The citric acid content of sodium citrate and ferric ammonium citrate is approximately the same.

The use of iron as an indicator of hydrogen sulfide formation, of course, is not new. Wilson,[3] Schunk,[4] Beckwith and Moser,[5] Levine, Vaughn, Epstein, and Anderson,[6] Sulkin and Willett,[7] ZoBell and Feltham,[8] and others have reported on the use of ferric chloride, ferric citrate, and ferrous sulfate, while ferric ammonium citrate has been used by the Difco Laboratories[9] as a hydrogen sulfide indicator in a peptone-carbohydrate medium for several years.

Substitution of ferric ammonium citrate for sodium citrate in the usual citrate media, however, lowered the pH sufficiently to cause hydrolysis of the agar upon autoclaving. Therefore, the composition of the medium was altered and the constituents so adjusted that the reaction was slightly above pH 7. This not only produced a medium stable to autoclaving, but also brought it into the sensitive range of the iron indicator system. Sulkin and Willett[7] and Hunter and Crecelius[10] have shown that ferric ammonium citrate reacts best as

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an indicator of hydrogen sulfide in an alkaline (pH 7.5) menstruum. As this synthetic medium, after inoculation, progressively becomes more alkaline with growth of the organism, the ferric ammonium citrate functions efficiently as an indicator well within its sensitive range.

The emended formula is as follows: magnesium sulfate, 0.02 per cent; sodium ammonium phosphate, 0.15 per cent; sodium chloride, 0.5 per cent; dipotassium phosphate, 0.1 per cent; ferric ammonium citrate (green), 0.3 per cent; sodium thiosulfate, 0.3 per cent; sodium sulfite, 0.04 per cent; agar, 2.0 per cent. The final reaction is slightly above pH 7 with no adjustment required. The medium should be stirred before and again after heating.

Agar Slants.—The medium is tubed and slanted to provide a generous butt. The organism (free of substrate) is stabbed through the medium to the bottom of the tube and then streaked over the surface of the slant. At 37° C. intense blackening of the medium first appears along the stab or in the water of condensation, or both, in about fifteen or sixteen hours and continues to spread until the entire butt is jet black. The surface growth on the slant is yellow against the whitish agar. This growth becomes orange as incubation continues and furnishes direct evidence of growth. Heavy inocula which refuse to grow on this medium do not assume this deep yellow color.

Contrary to the findings of Vaughn and Levine,[11] the concentration of agar seems to increase sensitivity of the medium to hydrogen sulfide. However, there is little or no increase in sensitivity above 1.5 per cent of agar.

In several respects, evidence of hydrogen sulfide formation in synthetic medium differs from that in conventional peptone media, as will be noted from Tables I and II. It is obvious, of course, that there will be no blackening of the medium if the organism itself cannot utilize citric acid as a sole source of carbon. This is illustrated by two of the hydrogen sulfide-forming members of the genus Salmonella, *Salmonella choleraesuis* (variety from Kunzendorf) and *Salmonella pullorum*, neither of which utilize citric acid as a sole source of carbon. Inoculation of *S. pullorum* into synthetic medium, to which dextrose had been added as a supplementary source of carbon, was unsuccessful. The organism still was unable either to initiate visible growth or to form hydrogen sulfide during an incubation period of sixty days.

Quite unexpected was the behavior of *Salmonella sp.* (variety from Newcastle). According to Bergey[12] and others, this organism does not produce hydrogen sulfide. Moreover, this particular culture has been tested a number of times over a period of three years on peptone-ferric citrate agar, on peptone-ferrous sulfate agar (Kligler's iron agar) and on peptone-ferric ammonium citrate agar without noting a single instance of hydrogen sulfide formation even after prolonged incubation.

It will be noted in Table I that this Newcastle strain produces a very strong 1 plus blackening of the agar along the stab in this synthetic medium. It is quite conspicuous when the stab is made between the glass and the agar. Control tubes of Kligler's iron agar inoculated simultaneously showed no trace of blackening. The reason for this may only be surmised at present. It may

TABLE I

SALMONELLA AND PARACOLON GROUPS

ORGANISM*	SYNTHETIC MEDIUM		KLIGLER'S IRON AGAR‡
	GROWTH	H_2S	H_2S
S. sp. (variety from Newcastle) (1)†	+	+	−
S. pullorum (5)	−	−	+++
S. choleraesuis (1)	−	−	−
S. choleraesuis (variety from Kunzendorf) (3)	−	−	++++
S. typhimurium (2)	+	++++	++++
S. sp. (Derby type) (2)	+	++++	++++
S. sp. (Bareilly type) (2)	+	++++	++++
S. hirschfeldii (1)	+	++++	++++
S. enteritidis (Gaertner) (1)	+	++++	++++
S. sp. (Panama type) (1)	+	++++	++++
S. anatis (Rettger and Scoville) (1)	+	++++	++++
S. sp. (Oranienburg type) (1)	+	++++	++++
S. sp. (Newport type) (2)	+	++++	++++
S. sp. (Senftenberg type) (1)	+	++++	++++
S. sp. (Give type) (2)	+	++++	++++
S. sp. (Montevideo type) (2)	+	++++	++++
S. sp. (Rubislaw type) (1)	+	++++	++++
S. sp. (Worthington type) (1)	+	++++	++++
S. sp. (Lexington type) (1)	+	++++	++++
S. sp. (Kentucky type) (1)	+	++++	++++
S. sp. (Bredeney type) (1)	+	++++	++++
S. sp. (Newington type) (1)	+	++++	++++
S. sp. (California type) (1)	+	++++	++++
S. sp. (Meleagridis type) (1)	+	++++	++++
S. sp. (Berta type) (1)	+	++++	++++
S. sp. (St. Paul type) (2)	+	++++	++++
S. sp. (Illinois type) (1)	+	++++	++++
"True" paracolon (6)	+	−	++++
"Pseudo" paracolon (3)	+	−	−
"Pseudo" paracolon (1)	−	−	−

*Organisms were typed and their identity confirmed by Dr. P. R. Edwards of Kentucky Agricultural Expermient Station.

†Numbers in parentheses represent number of different strains used.

‡Difco Laboratories Inc., Detroit, Mich.

TABLE II

ESCHERICHIA AND AEROBACTER GENERA

ORGANISM	SYNTHETIC MEDIUM		KLIGLER'S IRON AGAR†
	SURFACE GROWTH	H_2S	H_2S
Esch. coli (18)*	−	−	−
Esch. freundii (5)	+	+	+
A. aerogenes (12)	+	−	−
Coli-aerogenes "intermediates" (8)	+	−	−

*Numbers in parentheses represent number of different strains used.

†Difco Laboratories Inc., Detroit, Mich.

be that peptone media have a tendency to suppress hydrogen sulfide formation by this particular organism. On the other hand, Hunter and Crecelius[10] were able to show that many organisms, including *Esch. coli* and *Aerobacter aerogenes* not normally considered as producing hydrogen sulfide, were able to produce blackening of the agar when bismuth was used as a more sensitive indicator of hydrogen sulfide. The ability of this Newcastle strain to form hydrogen sulfide in twenty-four hours or less in synthetic medium again emphasizes[10, 11, 13] that the ability of an organism to form hydrogen sulfide is a function of the particu-

lar medium used, and that whenever hydrogen sulfide production by an organism is mentioned, it is important that the particular medium used be mentioned also.

All of the other Salmonella cultures in our stock collection (except *S. choleraesuis, S. choleraesuis* [variety from Kunzendorf], and *S. pullorum*) were able to utilize citric acid and to show dense blackening of the medium, usually by the fifteenth hour. No cultures of *Salmonella schottmülleri* or *Salmonella paratyphi* were available for testing on this medium. However, Simmons[2] reports *S. schottmülleri* (hydrogen sulfide-positive) as citrate positive and *S. paratyphi* (hydrogen sulfide-negative) as citrate negative.

Ten strains of paracolons were available for testing on this medium and on Kligler's iron agar. Six of them correspond to Dr. Edward's definition of the "true" paracolon and had been so classified by him. Two of these were of the Z_4 type. The remaining four were nonhydrogen sulfide-forming, sucrose- and salicin-fermenting strains designated as "pseudo" paracolons. All but one strain of the latter (DL-125) were able to utilize citric acid as a sole source of carbon. None of the "true" paracolons had fermented lactose in 264 hours, while all of the "pseudo" strains formed acid and gas from it after 72 to 192 hours.

All paracolon strains except one (DL-125) grew luxuriantly on the surface slant of the synthetic medium. However, only the "pseudo" strains were able to grow equally well *deep under* the surface. The "true" paracolons apparently have difficulty in utilizing citric acid anaerobically and this was reflected in meager growth along the stab (Table III).

TABLE III

ORGANISM	SURFACE GROWTH	SUBSURFACE GROWTH (DEEP STAB)	BLACKENING OF SYNTHETIC AGAR	BLACKENING OF KLIGLER'S IRON AGAR
Paracolon ("true")	+	Slight	−	++++
Paracolon ("pseudo")	+ or −	Profuse (or −)	−	−
S. choleraesuis	−	None	−	−
S. choleraesuis (Kunzendorf)	−	None	−	++++
S. pullorum	−	None	−	+++
S. Newcastle	+	Profuse	+	−
Other Salmonella	+	Profuse	++++	++++

Although the "true" paracolons are vigorous hydrogen sulfide producers on peptone media, all of them persistently have refused to produce any blackening of the synthetic medium in stab inoculations (see below for possible exception).

Esch. coli, of course, is unable to utilize citric acid as a sole source of carbon aerobically. However, there is some evidence that at least some strains apparently are able to grow to a slight degree just beneath the surface. This growth seems to be microaerophilic in nature rather than anaerobic and, in this respect, is similar to the habit of the "true" paracolons.

Esch. freundii not only utilizes citric acid, but also forms hydrogen sulfide.

The genus Aerobacter grows luxuriantly aerobically and anaerobically but does not blacken the medium either with or without agar. Occasionally, bubbles of gas appear in the agar into which *A. aerogenes* has been inoculated. This may

be demonstrated, also, with the liquid form of the medium in fermentation tubes. It has been found that some strains will produce from 5 to 10 per cent of gas, while other strains seem unable to produce any (Table IV).

TABLE IV

ORGANISM	FERMENTATION OF LACTOSE IN 24 HOURS	SURFACE GROWTH	SUBSURFACE GROWTH (DEEP STAB)	BLACKENING OF SYNTHETIC AGAR	BLACKENING OF KLIGLER'S IRON AGAR
Esch. coli	AG	−	S	−	−
Esch. freundii	AG	+	P	+	+
Intermediates	AG	+	S	−	−
A. aerogenes	AG	+	P	−	−
Paracolons ("true")	0	+	S	−	+
Paracolons ("pseudo")	0	Var. + or −	P (or −)	−	−

AG, Acid and gas.
O, No gas; slight or no acid.
S, Slight microaerophilic growth.
P, Profuse anaerobic growth.

Agar Plates.—While the foregoing work applies to agar slants, the medium may be used in Petri plates both for streak-stab inoculations and for "poured" plates. For both purposes a rather thick agar layer should be used (20 c.c.). For streak plates, the inoculum also should be stabbed through the agar to the glass floor of the Petri plate if it is desired to demonstrate hydrogen sulfide formation. Blackening will occur at the bottom of the stab or as black-centered colonies growing between the agar and the glass or both.

The medium is highly selective. Primary isolations may be made from direct smears or streaks from fecal or infected sources applied directly to the surface of agar films.

Unexpectedly, the Z_4 paracolon cultures readily have produced brilliant, jet black colonies between the agar and the glass in Petri plate streak-stab inoculations. These cultures persistently have refused to form any trace of blackening in test tube-stab inoculations. This refusal may be due to their microaerophilic growth in the test tube. In Petri plates this microaerophilic requirement may be satisfied. This phenomenon seems to apply only to these Z_4 strains. Apparently it is not shared by any of the other strains of "true" paracolons which steadily refuse to blacken the agar both in Petri plates and in the test tube.

On the contrary, the Newcastle strain of Salmonella, which consistently produces a 1 plus blackening of the agar in test tube-stab inoculations, does not produce any blackening of colonies in Petri plate cultures.

These two characteristics may prove of some value in the identification of these organisms (Table V).

TABLE V

ORGANISM	BLACKENING OF STAB IN TEST TUBE	BLACK CENTERED COLONIES IN PETRI PLATE
"True" and "pseudo" paracolons	−	−
Z_4 paracolon	−	+
S. sp. (variety from Newcastle)	+	−
Other Salmonella	+	+

Liquid Medium.—By omitting agar from the formula, the medium acts like Koser's citrate medium except that in addition to turbidity, there also is the orange reddening of the medium as an indication of growth. Hydrogen sulfide formation may be detected by a blackening of sediment at the bottom of the tube.

DISCUSSION

It is believed that this new ferric ammonium citrate medium will serve a useful and valuable purpose in several fields of bacteriology. The value of existing citrate media has been confined, principally, to differentiating *Esch. coli* from *A. aerogenes* and the coli-aerogenes intermediates. This new medium materially extends the usefulness of these media by providing a three-fold separation of (a) *Esch. coli* from (b) *Esch. freundii*, and of both of them from (c) *A. aerogenes* and the citrate-utilizing, nonhydrogen sulfide-forming coli-aerogenes intermediates.

Within the genus Salmonella, the usefulness of citrate media, in the past, has been restricted to a few species or varieties, such as *S. paratyphi, S. choleraesuis,* and *S. pullorum,* which are unable to utilize citric acid as a sole source of carbon. This new ferric ammonium citrate medium becomes an important differential medium for this genus. On it, for example, *S. sp.* (variety from Newcastle) is shown to produce hydrogen sulfide. It is believed that this is the first time detectable hydrogen sulfide formation may have been reported for this organism. Because of this characteristic, this medium offers a valuable means for differentiating *S. sp.* (variety from Newcastle) from the other Salmonella. The appearance of the hydrogen sulfide reaction differs so markedly from that of other Salmonella that little or no confusion is likely.

This ferric ammonium citrate synthetic medium used in conjunction with one of the conventional peptone-hydrogen sulfide indicator media should be of material aid, also, in the differentiation of *S. pullorum, S. choleraesuis, S. choleraesuis* (variety from Kunzendorf), and *S. paratyphi.*

A synthetic medium is described which demonstrates the presence or absence of detectable hydrogen sulfide produced by those bacteria capable of utilizing citric acid as a sole source of carbon.

It may be used in agar slants either as a plating medium or (in liquid form) as an alternative to Koser's citrate medium.

Bacteria either grow or do not grow on the medium, depending on whether they are able to utilize citric acid.

Those bacteria which do grow on it may or may not form hydrogen sulfide.

Intense blackening of the agar occurs within fifteen to twenty-four hours after inoculation.

The ability to form detectable hydrogen sulfide seems to be confined specifically to the Salmonella group of organisms and to *Esch. freundii.*

S. sp. (variety from Newcastle), previously considered as not forming hydrogen sulfide, consistently shows a 1 plus blackening of the agar within twenty-four hours in this medium. This should prove a valuable means of identifying this organism if this characteristic is found to hold true generally for all Newcastle strains.

The Newcastle strain which produces a 1 plus blackening in test tube slants fails to do so in Petri plate inoculations.

The paracolons, which readily form hydrogen sulfide in peptone media, fail to do so in test tube slants of synthetic medium.

The Z_4 paracolon, which does not produce hydrogen sulfide in test tube slants of synthetic medium, produces black colonies on streak-stab Petri plate inoculations.

Some of the Salmonella group which form hydrogen sulfide on peptone media do not grow on the synthetic medium. These two media used together provide evidence of value in differentiating various members of the Escherichia, Aerobacter, Salmonella, and paracolon groups.

It is believed that this may be the first instance where a synthetic or a non-peptone medium has been used to detect hydrogen sulfide formation by bacteria.

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THE APPLICATION OF A HOT WIRE AND THERMOCOUPLE FOR RECORDING SURFACE PULSATIONS IN THE HUMAN BODY*

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A GREAT deal of ingenuity has been demonstrated in the development of methods for recording arterial, venous, and other pulsations from the surface of the human body. The older sphygmographs (Wiggers, 1923[1]) employ a button, spring, or cup applied to the skin over an artery and transmit the movement to a lever system or to a column of air connected to a recording tambour. These have been largely supplanted, where accuracy of registration is desired, by instruments having a higher figure of merit. In one type of transmission instrument, the pulsations are picked up by a button, capsule, cup, or plethysmograph and led to a segment capsule or similar device, the movements of which are optically recorded (Wiggers, 1928;[2] Bolton, Carmichael, and Stürup, 1936;[3] Turner, 1937[4]). Pulses in a finger have also been obtained by transmission plethysmographs in which the movements of a drop of fluid (Johnson, 1932[5]) or of a water meniscus (Goetz, 1934,[6] 1940[7]) are photographed. Records of the pulse have also been made in terms of the change in resistance of a carbon microphone (Waud, 1924;[8] Turner, 1928[9]) or of a crystal microphone (Miller and White, 1941[10]) or by the change in electrical capacitance of a condenser unit (Fenning and Bonar, 1939;[11] Fenning, 1943[12]). The demonstration by Bonsmann (1934),[13] that a photoelectric cell is capable of detecting differences in the blood supply to a region, led to the development of photoelectric pulse recorders for regions such as the fingers, toes, ear, and nasal septum (Hertzman and Spealman, 1937;[14] Hertzman, 1937;[15, 16] Matthes and Hauss, 1938[17]). The change in electrical impedance of a region between electrodes with variations in blood supply has been used as the basis of a pulse-recording method by Mann (1937)[18] and Nyboer (1944).[19] Although not giving any results, Asher and Hopf (1935)[20] have devised an interesting arrangement whereby variations in blood supply to a region, such as the arm, result in an unbalance between the outputs of two vacuum tube oscillators, which unbalance, after rectification and amplification, is translated into the deflections of an oscillograph.

To minimize inertia, Hill (1921)[21, 22] used an air transmission system in which the movements of air resulting from each pulse cause a cooling of a very fine, heated wire, thus resulting in a change in resistance of the wire and unbalancing a Wheatstone bridge, of which the wire is a part. As Hill himself points out, this method is of value in determining certain time relations but it gives no accurate picture of the movements of the artery. Wishing to utilize

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the movement of a small mass of air, as Hill did, but hoping to improve the low frequency response of such a system, we have devised a simple arrangement employing a thermocouple and hot wire. Although this method does not give an exact representation of the pulse, it succeeds, as will be shown, in portraying the main components in their proper time relations.

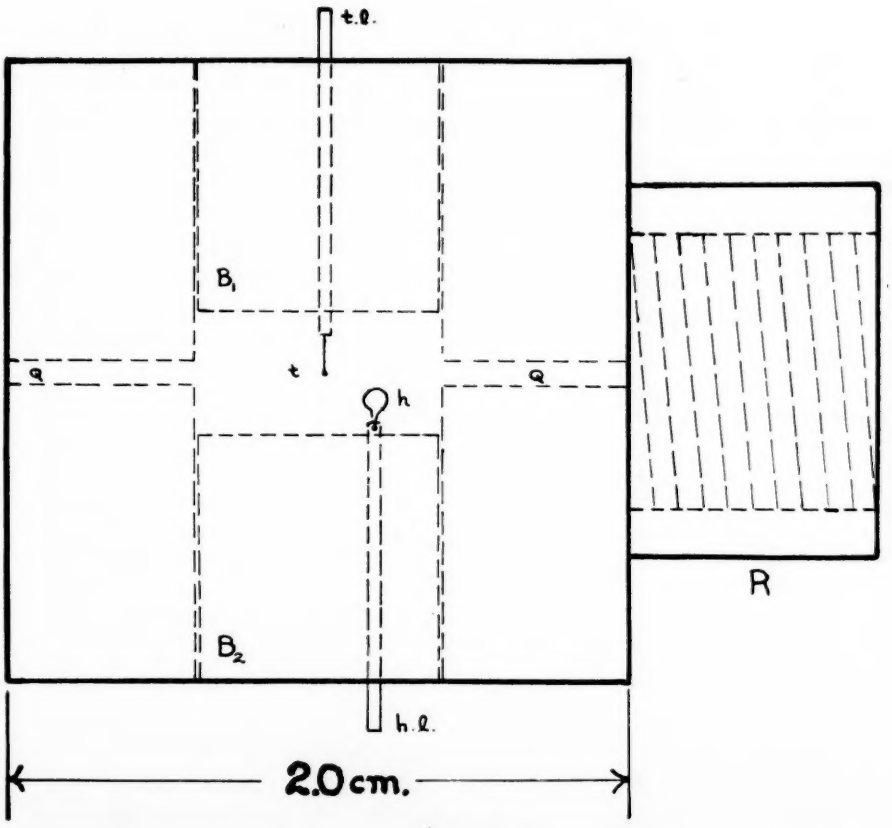
DESCRIPTION OF THE PULSE RECORDER

The unit (Fig. 1) consists of a lucite block with a transverse hole, into which are cemented two cylindrical lucite pieces (B_1, B_2). On one of these pieces is mounted a copper-constantan thermocouple, the diameter of the copper and constantan wire being 0.001 inch. On the other piece is placed a small helical coil (h) wound from 0.003 inch diameter nichrome wire. This coil is mounted so that it is aligned with one edge of a 1 mm. opening (Q) drilled longitudinally through the lucite block. The active junction (t) of the thermocouple is about 0.5 to 1.0 mm. away from the coil but aligned with the center of the opening (Q). The basic idea is that the coil, heated by means of an electric current, results in the formation of a small pocket of warm air in front of the active junction. The pulse then causes a movement of warm air against the active junction, resulting in a signal (E.M.F.) which is first amplified and then recorded oscillographically. The heat capacity of the active junction should be minimized by keeping the mass of the soldered joint as small as possible. The inactive junction is, of course, the junction between the constantan wire and one of the copper lead-off wires ($t.l.$). Its mass, and therefore heat capacity, is made large relative to that of the active junction, so that it will be influenced only by slow changes in temperature. One end of the lucite block is provided with a receiver (R) having an internal thread into which can be fitted any one of the adapters which are used in leading off from the pulsating surface. To obtain maximum sensitivity, these adapters should fit accurately so that no leaks exist. We have employed plaster of Paris molds of the surfaces to be fitted. Adapters for the finger, the neck (carotid artery), and the wrist (radial artery) are pictured in Fig. 1.

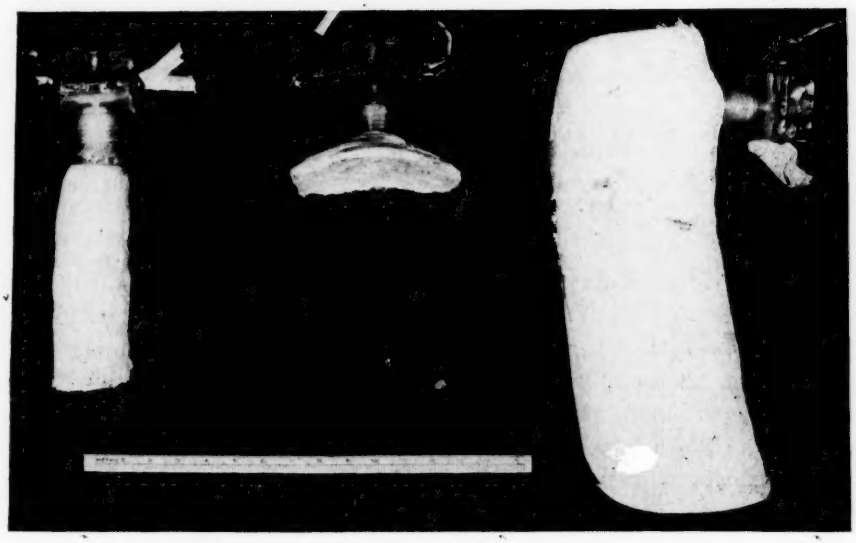
In actual practice the heating current for the coil is supplied by a 3.0 volt source. This is sufficient to bring the coil, whose resistance is 8 to 10 ohms, to a reddish glow. The relatively small signals (50 to 200 microvolts, records *T, U;* Fig. 2) are first amplified by means of a three-stage, high-gain, resistance-capacitance-coupled amplifier, of variable time-constant, and then fed into a cathode ray oscillograph. The maximum time-constant of 2.0 seconds (record *V,* Fig. 2) was always employed when accurate registration of the signal was desired. The entire instrumental problem can be greatly simplified if a high sensitivity galvanometer of adequate period, such as one of those employed in geophysical work, is available.

RESULTS

1. *Finger Pulses.*—It is our experience that the finger pulse is easily and quickly recorded with this method. For success it is essential to use a snugly fitting adapter in which the dead space is reduced to a minimum. The adapter



A.



B.

Fig. 1.—*A*, Diagram of the pulse recorder. The heating coil, *h*, is in reality a helix rather than a loop as shown here. Only one of the two thermocouple lead-off wires, *t.l.*, and only one of the two heating coil lead-off wires, *h.l.*, are illustrated. *B*, Photographs of the unit with a finger adapter, *left*, a carotid adapter, *center*, and a radial adapter, *right*. Further explanation in text.

can easily be made snug, yet nonconstrictive, by depositing several layers of plaster of Paris bandages over a thin rubber mold placed on the subject's finger. When the plaster has dried, the rubber is removed, leaving the finished adapter. Finger pulses from seven individuals are pictured in records *A* to *H*, Fig. 2. All of these were obtained from healthy adults except for record *H*, which represents the pulse from a 63-year-old patient with hypertension (blood pressure, 160/120) and slight arteriosclerosis.

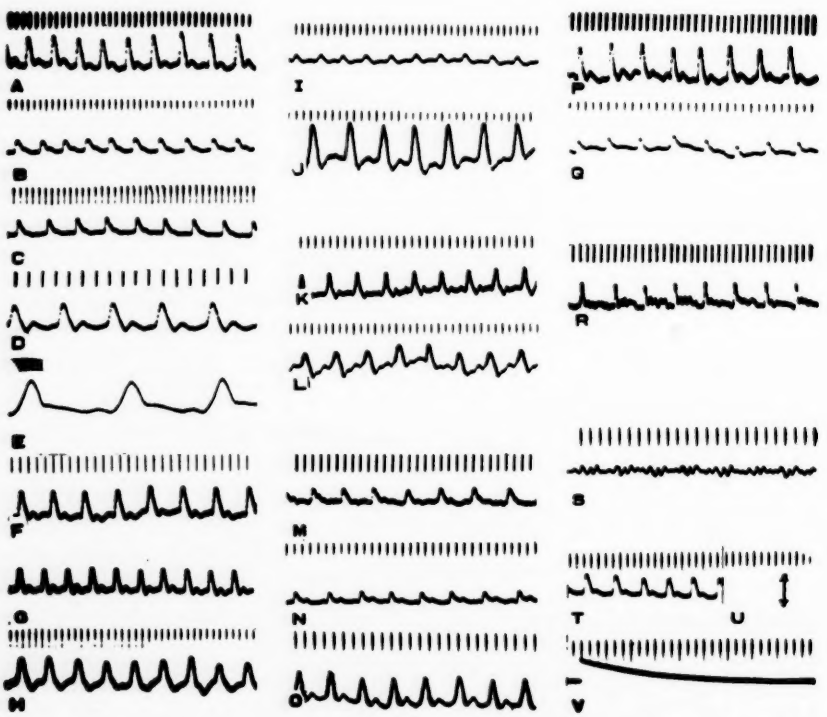


Fig. 2.—Photographs of records taken with the cathode ray oscillograph to show the pulse from various regions of the body. Records *D*, *E*, *I*, *J*, and *T* were all obtained from the same person (E. G.); the others were taken from different individuals. Record *T* is a finger pulse to be compared in magnitude with a record, *U*, showing a 100 microvolt, 60-cycle sine wave (arrow shows amplitude). Record *V* shows the response of the amplifier to the "on" of a square wave. The time line in all records indicates 0.2 second.

The wave form of these finger pulses was evaluated by comparing it with the form of the finger pulses obtained from the same individuals but using the photoelectric method. In Fig. 3 are shown tracings made from enlarged projections of the photographic records from three individuals. In this figure the finger pulses obtained with the present method (*B*) are compared with the finger pulses recorded by means of a phototube (*A*). The accuracy of registration of the finger pulse by the photoelectric technique has already been checked by Dillon and Hertzman (1941).[23] It will be seen that the primary wave, the dicrotic wave, and the dicrotic notch appear in the same time relations in the pulses obtained with both methods. The chief difference between the two pulse forms is that the catacrotic limb, instead of falling slowly as it should, descends

more rapidly in the thermoelectrically obtained pulse. In other words, the distortion is one due to the failure of the thermocouple to record slow changes. This leads to the appearance of the dicrotic wave much lower on the catacrotic limb than it actually should be, but the time relations are accurately reproduced.

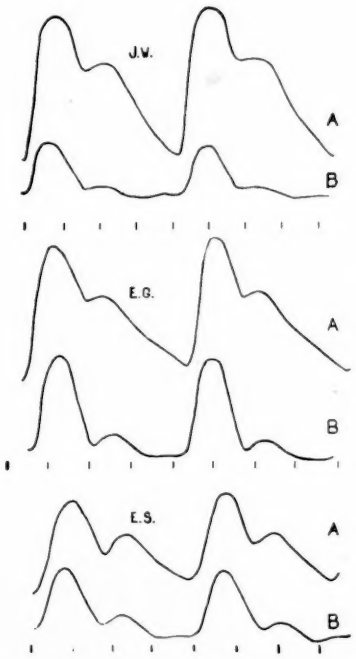


Fig. 3.—The finger pulse from three persons obtained thermoelectrically, *B*, compared with the same pulse recorded with a phototube, *A*. Time line indicates 0.2 second. Explanation in text.

A similar comparison of the pulses obtained by means of the thermoelectric method with the pulses recorded by Dillon and Hertzman (1941),[23] using the photoelectric cell, has led to the same conclusion that appears from a study of Fig. 3. The wave form may be further evaluated by determining the crest time; that is, the time to maximum of the primary wave (Table I). The mean figure for the digital crest time of normal subjects obtained by Dillon and Hertzman (1941),[23] using the photoelectric plethysmograph, is 0.127 second. The difference between the latter figure and that given as the mean in Table I is hardly a significant one.

TABLE I

FINGER PULSES

SUBJECT	CREST TIME (SEC.)
F. C.	0.114
J. M.	0.127
J. W.	0.125
E. S.	0.131
E. S. S.	0.119
G. S.	0.111
Mean	0.121

It is of some concern to inquire whether differences in details of construction are critical in the operation of the unit and in the results obtained with it. Six units were made according to the general diagram of Fig. 1. In these there are minor variations in the size of the active junction, the resistance of the active junction (3 to 5 ohms), the position of the active junction with respect to the heating coil, and the position of the heating coil with respect to the opening (*Q*). Tracings of the same finger pulse obtained with these six units (*A* to *F*) and with a phototube (*G*) are presented in Fig. 4. Aside from differences in sensitivity from one unit to another, the wave form for the different units is quite similar. Thus if reasonable care is employed, unavoidable variations in construction make no difference in the operation of the unit or in the results obtained through its use.

Another pertinent detail is the relation between the degree of heating of the coil and the operation of the unit, especially with regard to its sensitivity. In records *F* to *M*, Fig. 5, are presented some finger pulses taken with heating coil currents varying from 320 milliamperes (record *F*) to 150 milliamperes (record *M*). There is a diphasic distortion in these records due to the use of one of the shorter amplifier time-constants, but this does not influence the conclusion that for a considerable range of coil current the unit retains a constant

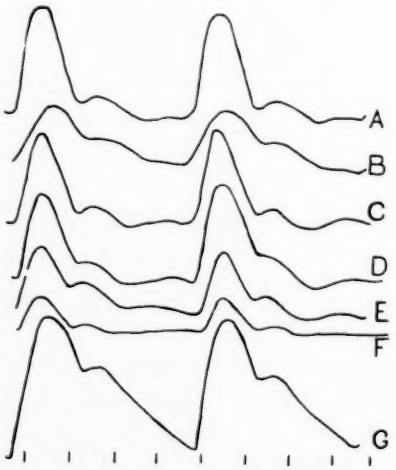


Fig. 4.—The finger pulse obtained from one subject using six different units, records *A* to *F*, and a phototube, *G*. The same amplifier gain was used throughout. Time line indicates 0.2 second. Explanation in text.

sensitivity and yields similar results. It is only when the coil current goes below about 250 milliamperes that a significant loss in sensitivity results. Since the unit is independent of the degree of heating of the coil over a wide range, the possibility of error due to inadvertent variations in the heating current is eliminated. This series of records also illustrates the need for a heating coil, since a thermocouple alone does not possess the same degree of sensitivity as does the combination.

Because of disturbances due to movements of the air, resulting from activity in the hand or fingers, it is essential to know whether the opening (*Q*) must pass

completely through the unit to the outside. To obtain an answer, the finger pulse was first recorded with the opening (*Q*) closed off by a small strip of adhesive (record *O*, Fig. 5). The strip was then removed and the normal finger pulse was obtained (record *P*, Fig. 5). It is obvious that sealing off the opening leads to reflected air waves and interferences which prevent the use of the unit as a pulse recorder. It might be possible to obtain a usable instrument by sealing a cup, the volume of which is large compared to the volume inside the unit, over the end of the lucite block. We have not attempted this, since it is not too difficult for the normal subject to maintain a steady position long enough to

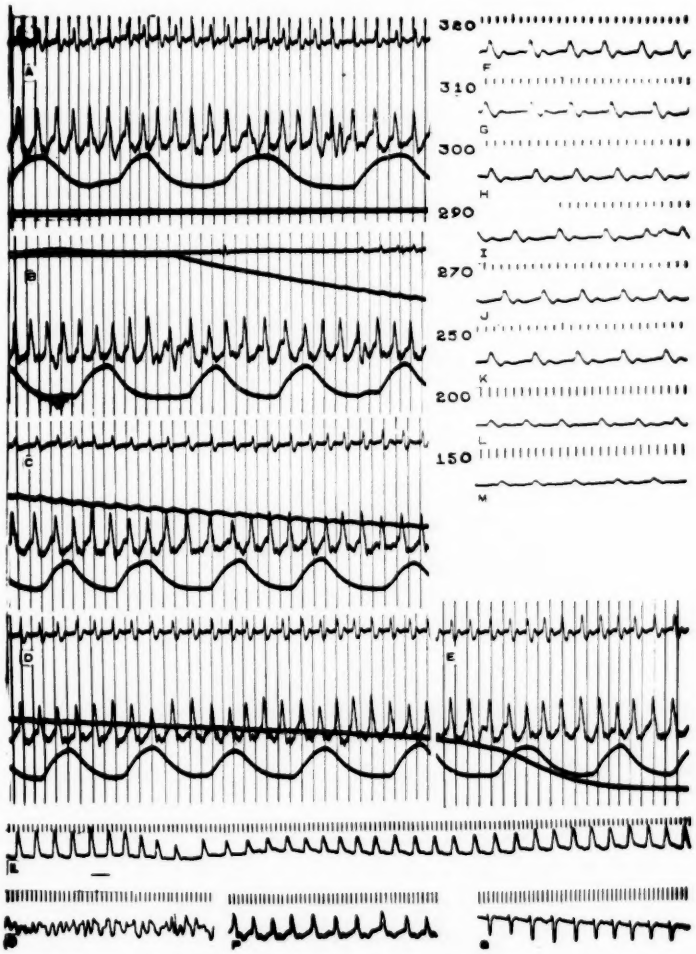


Fig. 5.—Record *A*; simultaneous multiple recordings of the finger pulse (upper), carotid pulse (next below), respiration (next below), and the cuff pressure (lowest). The two pulse records, which are distorted due to the use of too low an amplifier time-contact, were obtained using two resistance-capacitance-coupled amplifiers and two galvanometers from the Sanborn electrocardiograph. Respiration was recorded by means of a thermocouple mounted in a nosepiece, the variations in temperature with respiration being recorded with a Cambridge pot galvanometer. The cuff pressure was recorded by means of a stretched rubber membrane on which was cemented a small opaque vane in the path of a light beam to a barrier layer photocell, the output from the cell being fed into a recording microammeter. Record *A* was taken with the cuff pressure at zero. Record *B* shows the cuff pressure starting at 160 mm. of mercury but slowly being reduced. Records *C*, *D*, and *E* indicate the gradual fall in cuff pressure to zero. The time line in records *A* to *E* indicates 0.5 second. Record *N* shows the effect of one deep inspiration (dark line below) on the finger pulse. Time line in records *F* to *Q* indicates 0.2 second. Explanation in text.

record a considerable length of record, but some modification of this type might be necessary in recording the pulse from individuals with an uncontrollable tremor.

2. *Toe Pulse.*—The pulse from the toes is easily obtained with this method if the precautions mentioned in connection with the finger pulse are used in the construction and application of the toe adapter. A toe pulse taken with the same amplification as used in obtaining the finger pulses is shown in record *I*, Fig. 2. The same toe pulse using a higher amplification is given in record *J*, Fig. 2. There is some distortion in this record due to the necessity of having to increase the amplifier screen voltages in order to obtain the higher gain.

3. *The Pulse From Other Regions of the Body.*—The brachial, radial, carotid, and temporal pulses are somewhat more troublesome to obtain properly than are the finger and toe pulses. This is because of the difficulty of getting an adapter that will fit snugly and properly in relation to the pulsating surface and because of movements which produce artifacts and disturbances. The brachial pulse (*K, L*), the radial pulse (*M, N, O*), the carotid pulse (*P, Q*), the temporal pulse (*R*), and the apex beat (*S*) are presented in Fig. 2. For a comparison of the pulse wave form from various regions, tracings made from enlarged projections of the photographs are presented in Fig. 6. Records *A, B,* and *C* were obtained from the same person and show, respectively, the finger, the carotid, and

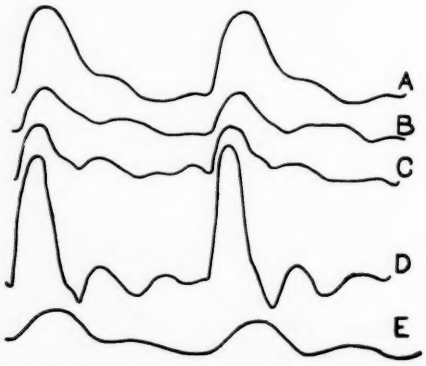


Fig. 6.—The pulse from various regions of the body compared. Explanation in text.

the radial pulses. The brachial pulse (*D*) was taken from a second individual, while record *E* is a toe pulse from still a third person. It appears from these tracings and from the actual records that not only the dicrotic wave but also predicrotic and postdicrotic components are brought out.

A poorly fitted adapter in regions such as the carotid, radial, brachial, or temporal may result in a deflection of reversed polarity. An example of this is indicated in record *Q*, Fig. 5, where a small upward deflection (in the usual direction) is followed by a main swing downward. Whenever this situation has arisen, it has been possible to correct it by either changing the position of the adapter or by replacing it with a better fitting one. Apparently in such cases a leak between the skin surface and the adapter results in the introduction into

the unit of room air which is cooler than the air normally within the unit. As a consequence there occurs, with each pulse, a cooling, rather than a warming, of the active junction.

DISCUSSION

It would appear from the introduction to this report that a sufficient number of adequate techniques for recording surface pulsations already exists and that another method is merely superfluous. It is debatable, however, whether a simple, portable, and accurate method capable of registering the pulse from various regions of the body is available. The methods employing segment capsules and optical registration are excellent for research work but are not convenient for use in a portable sphygmograph. Some methods, such as those of Asher and Hopf (1935)[20] and Nyboer (1944),[19] make use of relatively complex electronic circuits which might require repeated attention. The photoelectric method is ideal for recording the pulse from the pinna, the finger, and other regions where a sufficient amount of light is transmitted, but its use in such regions as the wrist and neck presents major difficulties. The methods of Johnson (1932)[5] and Goetz (1934)[6] make use of relatively simple and portable apparatus, but there is always the question of whether enough sensitivity is available for all circumstances. It is possible that by employing a photoelectric cell instead of photographing the movements of the drop or meniscus, an increase in sensitivity could be achieved, but the figure of merit of such a system should

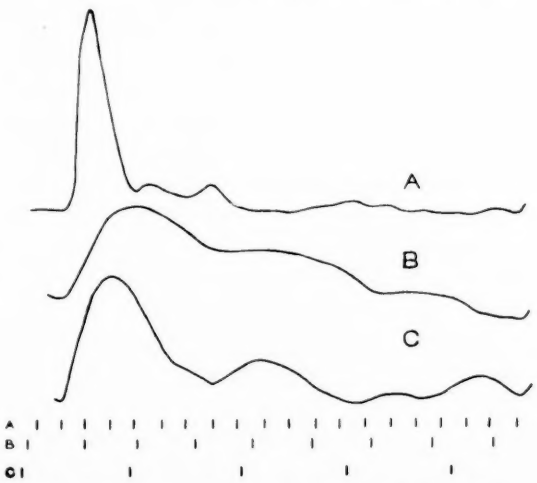


Fig. 7.—The radial pulse taken by three methods. *A* is a hot wire record, *B* is a Frank capsule record, and *C* is a record taken with the method described in this report. Time line for *A* is 0.04 second; for *B*, 0.1 second; and for *C* 0.2 second. Explanation in text.

be under suspicion until adequately checked. The techniques which employ a piezo-electric crystal (Miller and White, 1941[10]) and a capacity pick-up (Fenning and Bonar, 1939[11]) have not been fully tested, but they do offer interesting possibilities. The method of Hill (1921)[21, 22] is intriguing because of its simplicity and because it reduces problems of inertia to a minimum, but the pulse wave form obtained with the hot wire departs considerably from the true wave form. A comparison of the radial pulse taken by (1) the method of

Hill, (2) a Frank capsule, and (3) the thermoelectric method herein described is made in records *A, B, C,* respectively, of Fig. 7. Record *A* was reproduced from a figure given by Hill (1921),[22] while record *B* was traced from a record in the paper by Bazett and Dreyer (1922).[24] It will be seen that the primary deflection occurs very early in the pulse recorded with the hot wire and that the dicrotic wave is not indicated at all. These facts were recognized by Hill, who did not intend that his method be used in any way except in measuring pulse velocity and time relations in circumstances where knowledge of the true wave form is not necessary.

The present thermoelectric method shares, along with the hot wire technique, the advantage of a system with minimum inertia. It should be emphasized that it, too, does not give a truly exact picture of the pulse, although it does portray the main contours in their proper time relations. The method is extremely simple to use, especially when recording the finger pulse, and it may serve a useful function in problems where the low frequency distortion is of no consequence. Although we have employed a rather complicated amplifier coupled with a cathode ray oscillograph, there is no good reason why a fairly simple and stable amplifier feeding into a sensitive but rugged galvanometer could not be employed, thus making the entire apparatus compact and readily portable.

It might be of value to indicate some possible applications of the thermoelectric technique. Records *A* to *E,* Fig. 5, show the left index finger pulse, the carotid pulse, respiration, and the pressure in a cuff about the left arm. A measurement of the time displacement of the pulse waves in the carotid and finger pulses, a displacement which is clearly evident in the records, plus a measurement of the added distance traveled by the pulse in reaching the finger, should give a figure for the calculation of the pulse velocity. For the photographs in record *A,* the pulse velocity, when the above measurements were made, was 8.2 meters per second. The records (*B, C*) showing the appearance of the pulse as the cuff pressure is slowly reduced from 160 mm. of mercury indicates the possible use of this unit in blood presssure determinations. A record of the effect of one deep inspiration (record *N,* Fig. 5) indicates the ability of the unit to detect peripheral circulatory changes. It has been pointed out by a number of investigators (Dillon and Hertzman, 1941[23]) that the digital crest time is increased in such conditions as arteriosclerosis and hypertension. We have had occasion to examine one patient with hypertension; a record of the finger pulse is shown in record *H,* Fig. 2. The mean crest time for this pulse was 0.169 second, whereas the mean value for six normal subjects (Table I) was 0.121 second. This one observation, of course, has no statistical meaning, but it does indicate the need of examining the method further for possible use in cases where a change in configuration of the pulse wave occurs.

SUMMARY AND CONCLUSIONS

1. For the registration of the pulse from the surface of the human body a method is described which employs a heating coil and thermocouple. The method makes use of the E.M.F. developed across the two junctions of the

thermocouple as a small mass of warm air impinges upon the active junction with each pulse.

2. Records are shown of the pulse obtained from the fingers, toes, radial artery, brachial artery, carotid artery, temporal artery, and the apex beat. The precautions necessary for getting a successful record with this method, as well as some of the technical details connected with its operation, are pointed out.

3. The method has been evaluated by comparing the finger pulse obtained through its use with records of the finger pulse recorded by means of the photoelectric plethysmograph. It is shown that the primary pulse wave and the secondary waves are portrayed in their correct time relations. The digital crest time agrees well with figures previously published and obtained with the photoelectric technique. The chief limitation of the method results from its poor low frequency response, so that the catacrotic limb descends prematurely, causing the dicrotic notch and dicrotic wave to appear low in the record.

4. A number of possible applications for the technique are suggested, including its use in the measurement of the pulse velocity, its use as a unit in blood pressure determinations, and its possible employment in cases of disturbance in the time relations of the components of the pulse wave.

We wish to express appreciation to Dr. Gordon H. Scott for his help and encouragement throughout the progress of this work. To Mrs. Lois L. Shwartz are due our thanks for the construction of the photoelectric pressure recording unit mentioned in this paper, and to Dr. A. W. Martin of the University of Washington, Seattle, we are indebted for the loan of the oscillograph, the amplifier, and accessory pieces of equipment.

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REMOVAL OF BACTERIAL PYROGENS FROM PROTEIN HYDROLYSATES

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THE intravenous administration of amino acids has become of increasing importance in medicine within the last five years. Purified amino acids have been used for this purpose in some instances.[1, 2] The cost is high, however, and as a result the amino acids have usually been provided by protein hydrolysates.[3-6] The preparation of enzymatic[7, 8] and acid[9] hydrolysates of protein has been described, with casein the protein of choice. The nutritional quality and utilization of the final hydrolysates have been studied in laboratory animals.[7, 9, 10] An important requirement of such hydrolysates, in common with all substances for intravenous use, is freedom from bacterial pyrogens (fever-producing substances).[11] A method for removing pyrogen from crystalloidal solutions has been described,[12] but there have been no reports on the removal of pyrogen from protein hydrolysates. Procedures for determining the concentration of pyrogen and its reduction and eventual elimination from protein hydrolysates are reported herein.

EXPERIMENTAL

Determination of the Concentration of Pyrogen.—A standard test for the presence of bacterial pyrogen is now available;[15, 16] however, the range of concentration of pyrogen over which a proportional temperature rise is obtained in the rabbit is too short to be utilized for quantitative purposes. Typical data for the temperature rises obtained with various amounts of purified pyrogen are shown in Table I. Similar data have been published by others.[13] Recently the leucocytosis accompanying the injection of pyrogen was reported[14] to be approximately proportional to the amount of pyrogen. However, the data show that the proportionality is no better than that indicated by the temperature rise.

The concentration of pyrogen was determined from the minimal reactive dilution.[18] This phase of the study was facilitated by a preparation of a purified pyrogen, helpful both for determining the amount of pyrogen and the capacity of filters to remove pyrogen. From the data in Table I and other comparable experiments it was concluded that approximately 0.01 μg. of pyrogen will give a perceptible temperature rise in rabbits. In testing for the pyrogen content, protein hydrolysates are diluted until an equivalent perceptible temperature rise is obtained on injection into rabbits. The pyrogen content can readily be calculated from the dilution of the hydrolysate and the minimal reactive dose of pyrogen of 0.01 μg. The results indicate only roughly the amount of

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pyrogen involved since the standard of comparison is not completely purified and differences in the reactivity of pyrogen from different bacterial sources have been reported.[18]

Three to five rabbits were used for each test. The amounts of the substances indicated are the dosages per kilogram of weight. The volume of the purified pyrogen solution injected was 1 c.c. per kilogram; of the hydrolysate, 10 c.c. per kilogram.

The purified pyrogen was prepared by a modification of the method of Welch and co-workers[15] from *Pseudomonas aeruginosa*.

The pyrogen content of an enzymatic protein hydrolysate is shown in Table I. It contains over 0.01 μg. of pyrogen per milligram of hydrolysate. Other enzyme preparations have contained as much as 0.01 μg. in 0.1 mg. In repeated determinations with the same type of preparation, tests with only two or three dilutions were sufficient to establish the amount of pyrogen present. A large number of tests has been performed with the acid hydrolysates. The limitations of these data are shown by a typical experiment in which 5 mg. of hydrolysate gave a minimal reaction whereas 3 mg. did not.

TABLE I

TEMPERATURE RISE PRODUCED IN RABBITS BY A PURIFIED PYROGEN AND BY A PROTEIN HYDROLYSATE (ENZYME)

PURIFIED PYROGEN (μG)	TEMPERATURE RISE (° C.)	PROTEIN HYDROLYSATE (ENZYME) (MG.)	TEMPERATURE RISE (° C.)
1000	2.0, 1.8, 1.1*	500	1.6, 1.2, 0.8
100	2.3, 2.9, 2.2	50	1.8, 1.7, 1.6
10	1.5, 2.3, 1.8, 1.6, 1.2	25	1.3, 2.0, 1.1
1	1.0, 2.4, 1.7	10	0.9, 0.6, 0.3
0.1	0.6, 1.5, 0.9	5	0, 0.7, 0.9
0.01	0.80, -0.1, -0.1†	1	0.3, 1.2, 0.9†
0.001	0.5, 0.4, 0.3†	0.1	0.3, 0.4, 0†

*Two rabbits of this group died.
†A temperature rise exceeding 0.6° C. is indicative of pyrogens.[15-17]

Preparation of Protein Hydrolysates.—To obtain a pyrogen-free enzymatic digest, it was concluded on the basis of comparison of enzymatic hydrolysates of casein and human globin that the protein used should be essentially free of pyrogens. A pyrogen-free preparation was obtained from human red cells, collected aseptically and digested with fresh beef pancreas, precautions being taken to maintain sterile conditions throughout. Studies with this material were discontinued, however, because human globin is a deficient protein[19] and its availability is uncertain. Attempts to use this method with casein were unsuccessful because of its initial high content of pyrogen which could not be removed in subsequent operations. A small amount of pyrogen can be removed by Seitz filtration from water and from amino acid solutions, but it is difficult to accomplish when peptides are present as in enzymatic hydrolysates.

Since pyrogen-free proteins are not readily available, recourse was had to hydrolysis with acid, which destroys the pyrogen. Acid hydrolysates can readily be prepared by methods in general use[20] or those devised for preparing hydrolysates for bacterial nutrition.[21] Alkaline hydrolysates[9] were not considered desirable because of the racemization of the amino acids and the destruc-

tion of some of them. Since acid destroys all of the tryptophane, this essential amino acid must be added to acid hydrolysates. After the addition of tryptophane, preparations were tested for their ability to support the growth of rats,[22] since recent studies have indicated that the human requirements are similar to those of the rat.[23, 24]

After the acid used for hydrolysis was removed, bacterial contamination was avoided, as far as possible, to prevent the re-entry of pyrogen. The final hydrolysates were tested for pyrogens, the small amount of pyrogen usually present (about 0.01 μg. in from 2 to 5 mg.) being removed by Seitz filtration. After filtration the pyrogen content was less than 0.01 μg. per 100 mg. of hydrolysate; that is, 10 c.c. per kilogram of a 10 per cent solution gave no reaction in a rabbit.

A typical, dried casein hydrolysate contained 12.5 per cent total nitrogen, 66.2 per cent of which was amino nitrogen, 4.2 per cent moisture, and 2.3 per cent ash.

Removal of Pyrogen: The bacterial pyrogens are extremely soluble substances which appear to be polysaccharides.[12, 13, 15] In one case they have been reported to be nitrogen-free.[13] The purified pyrogen which we have isolated also has been largely polysaccharide in nature. Further study will probably provide a specific means for the destruction or removal of pyrogens from solutions, although none is available at present. None of the various methods tried for the removal of pyrogen has been adequate when a large amount of pyrogren was present. Filtration through asbestos pads (Seitz or Ertel) by the method of Co Tui and Wright[12] has been successful when the amount was small.

TABLE II

REMOVAL OF PYROGEN FROM VARIOUS SOLUTIONS AND PROTEIN HYDROLYSATES BY FILTRATION THROUGH A SEITZ PAD

SUBSTANCE	PYROGEN CONTENT (μG. PER 1,000 C.C.)	AREA OF SEITZ PAD* (SQ. CM.)
Purified pyrogen† in water	5,000	648
Casein hydrolysate (acid), 10%	300	65
Casein hydrolysate (enzyme), 10%	10,000	26,000
Purified pyrogen in 10% pyrogen-free casein hydrolysate (acid)	1,000	> 648

*In actual practice the volume of solution which could be freed of pyrogen by passage through two pads 20 cm. in diameter (648 sq. cm.) was determined.

†A temperature rise exceeding 0.6° C. is indicative of pyrogens.[15-17]

A summary of the results of the removal of pyrogen by filtration is given in Table II. Purified pyrogen, 5,000 μg. dissolved in 1,000 c.c. of water, was removed by filtration in an Ertel filter[13] through two S-3 Seitz pads (648 sq. cm.). Additional experiments showed that this was the absorptive capacity of the pads. Each square centimeter of pad removed about 10 μg. The Ertel No. 0 pads were no more effective than the Seitz pads. Co Tui and Wright[12] reported the removal of 100 μg. of a less active pyrogen per square centimeter of the Ertel pad. Unfortunately these data were not directly applicable to protein hydrolysates. The pad area required to remove equivalent pyrogen from enzyme hydrolysates was at least twenty times as great as that required for pure pyrogen in water. The area required for acid hydrolysates was almost the same as for pyrogen

removed from water. In some experiments larger areas were required than for the example shown in Table II. That this was due to the amino acids and not to a difference in the properties of the pyrogens was shown by the experiment in which the purified pyrogen was added to an acid hydrolysate previously freed of pyrogen. Filtration was much less effective than with the equivalent amount of pyrogen added to water. The difference between acid and enzyme hydrolysates was probably due to the presence of polypeptides, since the former is 100 per cent hydrolyzed, whereas the latter is only 63 per cent hydrolyzed.

DISCUSSION

The method described for the quantitative determination of pyrogen is time consuming. Further study of the bacterial pyrogens may reveal specific properties on which a more rapid determination can be based. Such knowledge also may reveal more satisfactory means for their destruction or removal. From a practical standpoint, the quantitative removal of small amounts of pyrogens from complete hydrolysates is feasible, but their removal from partial enzyme hydrolysates is impractical. In the use of large filtering areas the possibility of loss of essential amino acids must be kept in mind.

SUMMARY

A technique based on the minimal reactive dilution is described for determining the bacterial pyrogen content of protein hydrolysates. The test is used in the preparation of protein hydrolysates (acid) which are freed of pyrogen by means of Seitz filtration. Results showed that partial hydrolysates (enzyme) could not be freed of pyrogen by Seitz filtration.

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THE TITRIMETRIC DETERMINATION OF POTASSIUM IN TUNGSTIC ACID FILTRATES

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INTRODUCTION

THE basis of several methods[1-4] for the estimation of potassium in serum and plasma has been its precipitation by the addition of the cobaltinitrite ion to the preparation. However, in such procedures a mixture of insoluble salt complexes, namely, $K_2NaCo(NO_2)_6$ and $K_3Co(NO_2)_6$, is almost always formed which necessitates the determination of a conversion factor to obtain the true values for the amount of potassium in the precipitate. In the method to be reported in this communication it has been found that the potassium is quantitatively combined with the cobaltinitrite ion, and evidence is presented which indicates that the ratio between the potassium and the nitrite portions of the molecule is a constant quantity if the directions given for the determination are carefully followed. Consequently the titrimetric estimation of the nitrous acid by potassium permanganate gives a simple and accurate method for the measurement of potassium in the cobaltinitrite complex.[1, 2]

Although the complex of potassium and cobaltinitrite is soluble in tungstic acid filtrates, precipitation is essentially quantitative in the presence of 30 per cent ethyl alcohol. The method to be described in this report makes use of this fact, and therefore it is possible to determine potassium directly in tungstic acid filtrates of serum and plasma, as well as to determine other constituents such as chlorides or nonprotein nitrogen in aliquots of this same filtrate. In previous methods using tungstic acid filtrates, the cobaltinitrite was estimated colorimetrically.[3, 4] Since the potassium is determined titrimetrically in this procedure, the apparatus employed is simple and can be found in almost any clinical laboratory. With as little as 1 c.c. of serum or plasma, estimations of potassium may be made in triplicate. The method is relatively rapid and lends itself readily to routine clinical analyses; it has been used for this purpose with satisfactory results in our laboratory for the last year.

REAGENTS

1. Stable tungstic acid solution.[5]
2. Sodium cobaltinitrite reagent.[1]
3. Ethyl alcohol, 95 per cent.
4. 0.01 N potassium permanganate, prepared by dilution just before use from a stock 0.10 N solution which is made by dissolving 3.158 Gm. pure potassium permanganate in 100 c.c. water.

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5. 0.01 N sodium oxalate prepared by dilution just before use from a stock 0.1 N solution which is made by dissolving 6.700 Gm. sodium oxalate (Sorenson's analytical reagent, dried over calcium chloride) in 1 liter of water.

6. 4 N sulfuric acid.

METHOD

Precipitation of Protein.—The protein in 1 c.c. plasma (or serum) is precipitated by adding 9 c.c. of stable tungstic acid reagent and allowed to coagulate for approximately thirty minutes, then centrifuged for twenty minutes at 1,500 r.p.m.

Precipitation of Potassium.—A 2 c.c. aliquot of the clear supernatant fluid (equivalent to 0.2 c.c. of plasma) is added to a clean* centrifuge tube containing 1 c.c. 95 per cent ethyl alcohol. After mixing well, the solution is allowed to stand for five minutes to allow any microbubbles to disappear before adding 1 c.c. of the filtered sodium cobaltinitrite reagent slowly, drop by drop, agitating the mixture after each addition. After standing from forty-five to sixty minutes, the contents are centrifuged for twenty minutes at approximately 1,500 r.p.m. The clear supernatant fluid is sucked off slowly, using a fine bore tube drawn out to a J-shaped capillary, leaving about 0.2 c.c. of the fluid above the well-packed precipitate. The first portion of the 5 c.c. wash water is allowed to slide slowly down the side of the tube to prevent any disturbance of the precipitate and to rinse down any particles of the precipitate which might be adhering to the walls. Finally, the last portion of the water is used to wash the capillary pipette. To ensure mixing, the tube is then rotated carefully between the palms of the hands until the contents are a uniform color. The washing process is repeated four times with no more than from five to ten minutes of centrifuging between washes.

Titration.—The potassium permanganate solution is standardized just before use by adding 2 c.c. permanganate to 1 c.c. 4 N sulfuric acid and heating for from thirty to forty-five seconds in boiling water. A 2.5 c.c. sample of the standard 0.010 N sodium oxalate is added and the tube is heated in the water bath again and the excess oxalate titrated to a permanent pink color with the permanganate to be standardized. A blank, 0.025 c.c., the amount of permanganate necessary to give 1 c.c. of 4 N sulfuric acid added to 4 ml. water a permanent pink color, is subtracted from the total permanganate used. The normality of the permanganate is calculated by dividing the amount of permanganate required to neutralize the oxalate by the amount of oxalate employed (2.5 c.c.) and multiplying the result by 0.01.

For normal plasma (or serum), 2 c.c. of potassium permanganate are added to the precipitate; after intimately mixing with a fine-tipped glass rod, 1 c.c. 4 N sulfuric acid is added, rinsing down the glass rod at the same time. The tube is then placed in gently boiling water for from thirty to forty-five seconds. At the end of this time all of the precipitate should be dissolved and the solution a clear pink color. Either 1 or 2 c.c. of 0.010 N sodium oxalate, depending upon the amount of potassium in the sample, is added while the tube is still very hot. The contents of the tube are well mixed and then heated up in the

*The centrifuge tubes may be cleaned with a warm 2 per cent solution of "Orvus" followed by thorough rinsing with distilled water. This removes all traces of grease which would tend to trap small amounts of the fine precipitate and thus increase the possibility of losing some of it during the washing procedure.

boiling water for at least one minute before titration with the 0.01 N potassium permanganate to a permanent pink color, using a 1 c.c. microburette or a 1 c.c. pipette with a very fine tip and graduated in hundredths.

Calculation of the Factor for Converting the Titration Value to Milligrams of Potassium per 100 c.c.—The addition of acid to the precipitate liberates the nitrite portion of the complex as nitrous acid. The reaction with permanganate then proceeds as follows:

$$5\ HNO_2 + 2\ KMnO_4 + 3\ H_2SO_4 \rightarrow 5\ HNO_3 + K_2SO_4 + 2\ MnSO_4 + 3\ H_2O$$

If the permanganate is exactly 0.01 Normal, the amount of potassium is then given by the equation: $([(a-b)-d] \times f)$ mg., where

a = the actual volume of permanganate required in the analysis
b = the blank (0.025 c.c.)
d = the amount of standard 0.010 N sodium oxalate added.
f = the factor for converting the titration value to milligrams of potassium per 100 c. c.

In Table I is shown how the value for f was obtained from the determination of potassium in solutions containing amounts ranging from 62.8 mg. to 10 mg. potassium per 100 c.c. when the normality of the permanganate was exactly 0.01. However, if the permanganate is not exactly 0.01 N, but has a normality of N^1, the fraction $\frac{N^1}{0.01}$ should be applied to the volume $(a-b)$ of permanganate used. The milligrams of potassium per 100 c.c. of the sample analyzed by this procedure is therefore: $([(a-b)\frac{N^1}{0.01}] - d)\ f$

TABLE I

CALCULATION OF THE CONVERSION FACTOR f

(1) STANDARD SOLUTION OF K ADDED AS KCl MG. PER 100 C.C.	(2) $\left[(A-B)\frac{N^1}{0.01}\right] - D$	$\frac{(1)}{(2)}$ f
62.8	1.951	32.2
47.1	1.447	32.5
47.1	1.390	33.9
40.0	1.219	32.8
40.0	1.200	33.3
35.3	1.082	32.6
31.4	0.997	31.5
23.6	0.736	32.1
23.6	0.745	31.7
23.6	0.715	33.0
20.0	0.621	32.0
20.0	0.619	32.3
20.0	0.662	30.2
20.0	0.614	32.6
20.0	0.604	33.1
15.7	0.480	32.7
15.0	0.460	32.6
15.0	0.478	31.4
11.8	0.356	33.1
11.8	0.358	33.0
10.0	0.306	32.7
	Average	32.5 ± 0.8

RESULTS

Using the value of f = 32.5, single analyses were done on a sample of dog plasma to which were added known amounts of potassium chloride with the results shown in Table II. It is possible, therefore, to recover potassium quantitatively when it has been added to dog plasma. The average difference between duplicates of forty-two determinations in which this procedure was used amounted to 0.5 mg. per 100 c.c. plasma. Therefore the level of potassium in the sample analyzed should be within 3 per cent of the true value.

TABLE II

RECOVERY OF POTASSIUM ADDED TO DOG PLASMA

K FOUND (MG. PER 100 C.C.)	K ADDED (MG. PER 100 C.C.)	RECOVERY (MG. PER 100 C.C.)	DIFFERENCE (MG. PER 100 C.C.)
19.1	0.0	0.0	
23.1	4.0	4.0	0.0
23.2	4.0	4.1	+0.1
43.6	25.1	24.5	-0.6
44.2	25.1	25.1	0.0

In a series of thirty normal dogs the value for plasma potassium was found to vary between 13.0 and 19.8 mg. per 100 c.c. with an average value of 15.9 mg. per 100 c.c. Manery and Solandt,[6] using a slight modification of the Shohl and Bennett potassium method as described by Fenn and co-workers,[7] found that the concentration of potassium in plasma of forty-four normal dogs ranged from 12.2 to 20.0 mg. per 100 c.c. with an average value of 15.9 mg. (probable error, ± 1.7 mg.).

SUMMARY

1. A titrimetric method for the estimation of potassium in tungstic acid filtrates of serum or plasma has been described. The procedure is also applicable to lymph and ascitic and cerebrospinal fluid.

2. Determinations may be done in triplicate on 1 c.c. of serum or plasma.

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A COLORIMETRIC METHOD FOR DETERMINING AVAILABLE OXYGEN IN BLOOD

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IN PLANNING a clinical study of pulmonary anoxemia, one of the requirements was the collection of data showing the effect of different test conditions on the oxygen content of arterial blood. This requirement seemed to call for repeated arterial punctures, a procedure which is objectionable, if not impracticable. There remained only two alternatives: to use the so-called venous-arterial blood samples obtained from a vein after immersing an arm in hot water to speed up the circulation[1] or to use capillary blood samples from a pricked finger.[2] We used capillary blood samples in our determination of carbon dioxide[3] and found this entirely satisfactory. An inquiry into the differences of the oxygen content between arterial and venous blood under given controlled conditions was also contemplated, so the collection and care of venous blood has also been carefully considered. If the blood taken from the cubital vein or from a vein near the knuckles of the hand after immersion in hot water or from a finger prick is to remain a true sample for an oxygen content study, it must be protected from the atmosphere. With a finger-tip prick this was readily done by immersing the bleeding finger under oil.[3] The venous blood may be drawn with a syringe containing a little oil or attach the needle to a short rubber tube, the other end of this tube in turn attached to a glass tube running to the bottom of oil in a test tube, as suggested by Van Slyke and Cullen.[4] It was not considered advisable to allow any oil in a needle to be introduced intravenously into a human subject, so in collecting venous blood we took 6 c.c. in the usual way, but the needle point was always very promptly brought under the surface of the oil in a sample vial and 4 c.c. of the blood slowly expelled. The 2 c.c. withheld in the syringe were discarded on the assumption that the first portion entering the syringe would gather and hold all the atmospheric oxygen in the needle bore and the space between plunger and walls of the syringe. This is undoubtedly true in part, but the rush of the current from the very small orifice of the needle overcomes the plasma viscidity and carries through to the end of the plunger, thoroughly mixing the sample. There is also the matter of contamination from the oxygen dissolved in the oil. This will be taken up in the discussion of Table II.

Ferrous iron is very readily converted into ferric iron by molecular oxygen in a faintly alkaline menstruum but not at all in one that is acid. Ferric iron is very readily measured by converting it into any one of a large number of colored compounds. From an extended laboratory survey not only of iron, but also of other easily oxidizable substances, ferrous sulfate in a borax

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solution followed by sulfosalicylic acid was chosen as the most likely way to determine colorimetrically the oxygen in the blood both conveniently and quickly and on small samples such as could be obtained from a finger prick. Sulfosalicylic acid precipitates the blood proteins, getting them out of the way, and also combines with the ferric ion, giving a soluble compound which imparts a characteristic, stable, and consistent color to the solution with a color density directly related to the amount of ferric iron involved.

We now determine the available oxygen in the blood by mixing 0.1 c.c. blood sample with 0.1 c.c. ferrous iron solution in 1.5 c.c. borax solution, then introducing this mixture into 2 c.c. 30 per cent sulfosalicylic acid, making it up to 10 c.c. with water, filtering through iron-free paper, and measuring in a calibrated scopometer[5] the density of the color which develops. The oxygen content is then found opposite the shutter scale reading on a calibration chart.

EXPERIMENTAL

Reagents.—The required alkalinity in this test is not confined to a narrow range of pH, and the anions controlling the pH need not be limited to those of borax. Many alkalies in various concentrations have been successfully used, but we have finally settled on the convenient routine of 1.5 c.c. of 20 Gm. borax ($Na_2B_4O_7 \cdot 10H_2O$) per liter of water. The borax solution is fully aerated at atmospheric pressure and room temperature. The Fe++ reagent is added to the borax solution immediately after the blood sample. This reagent, likewise, need not be narrowly confined to a given concentration or anion, and in our laboratory it is prepared as follows: 500 mg. $FeSO_4 : H_2O$ are dissolved in 10 c.c. water, a little celite (inert filter aid) is added, and the solution is brought to a boil and kept simmering a few moments; it is then poured into a conical centrifuge tube, covered with mineral oil, and centrifuged. This reagent under oil is ferric-free and will remain so for days. However, it is well to make it up fresh each day an oxygen determination is done since the possibilities of Fe++ oxidation to Fe+++ by air contamination are considerable.

Anticoagulants.—Most blood comes to the laboratory with oxalate added as an anticoagulant. These samples are proper for acid hematin and the manometric procedures but give faulty values by the new colorimetric method since the C_2O_4 ion interferes with the oxidation of iron. Fluorides were also found unsatisfactory, sometimes depressing the oxygen values 20 per cent. The only anticoagulant found which was at all suitable proved to be heparin. The heparin used comes in 100 mg. vials.* A 1 per cent stock solution is made up and kept in the cold chest; this is rediluted as needed to measure out 0.3 mg. per 5 c.c. blood sample conveniently.

Apparatus.—It is obvious that during the interval when the oxidation of the Fe++ takes place no atmospheric oxygen must reach the reaction mixture. This we failed to accomplish in the ordinary test tube, using a layer of mineral oil to exclude the air, but we now do it without oil very conveniently by exchanging the test tube for a closed reaction chamber with a funnel cup joined to it by means of a stopcock to admit the sample and reagents and a rubber tube with mercury and mercury-levelling bulb below by way of another stopcock

*From the Connaught Laboratory of Toronto University.

for controlling the flow of fluids between the cup and the chamber. Fig. 1, a drawing of the apparatus, is almost entirely self-explanatory. The capacity of the reaction chamber is 3.5 c.c., and of the cup, 5 c.c., the latter calibrated to 4 c.c. in 0.5 c.c. intervals from the stopcock up. The cup is 65 mm. long and 16 mm. in diameter. The contraction of the funnel cup along toward the stopcock by which it is joined to the reaction chamber forms a 2 mm. capillary bore about 7 mm. long. The two stopcock barrels are 38 mm. long and have a 15 mm. inner diameter across the wider end. The stopper projects some 10 mm. at the tapered end and has a groove just adjacent to the cock over which a rubber washer is slipped to keep the stopper from coming loose unexpectedly. The bore

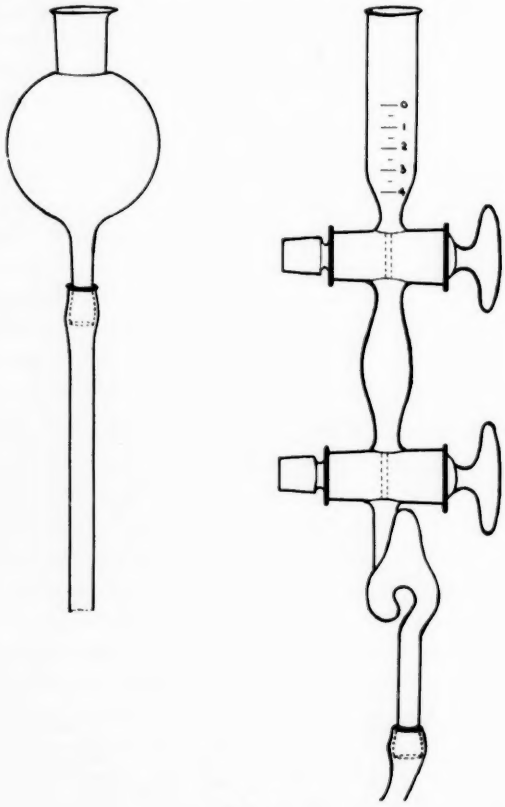

Fig. 1.

of the stopper is 2 mm. It is important that the upper stopper does not form a ledge upon which the blood may lodge; the bore should, therefore, be the same as the exit orifice of the cup capillary or a trifle more. The orifice of the upper stopper bore must not be bevelled. The glass tube by which the rubber tube is attached to the lower stopcock is looped and the top of the loop is expanded to form a trap (10 by 12 by 12 mm.) which catches any gas that may accidentally enter the mercury. Two seats are provided for the levelling bulb: in the upper seat the mercury is allowed to come to about the 1 c.c. mark in the cup; in the lower seat the mercury comes into the taper just above the lower stopcock.

It is very important that the apparatus be as clean as possible. It may be cleansed with water, alkali, or acid, or sometimes with chromic-sulfuric clean-

ing mixture, by drawing the cleaning fluid down through the cup into the reaction chamber, even to the trap if necessary, and then forcing it up again into the cup, all by changing the level of the mercury reservoir. The cleaning fluids must not add ferric iron as a contaminant. If the chamber is smeared with blood residues, it is most easily cleaned with a strong alkali, for instance, 2 c.c. 10 per cent NaOH, raising and lowering the levelling bulb so that the mercury will scrub the objectionable matter into the cup, from which it is flushed out with water by a pipette with a rubber bulb, or by the suction of a water pump. The apparatus must be free from protein, fat, and grease smears since these occlude oxygen and it takes only 0.001 c.c. oxygen to make an error of 1 volume per cent. The alkali should be rinsed out completely with iron-free water and finally with very dilute acid (0.05 *N* H_2SO_4). When the apparatus is put away, it should be filled with this dilute acid with the lower stopcock closed, the upper open, and the levelling bulb in its lower seat.

PROCEDURE

To determine the oxygen capacity of the blood sample, assure the complete saturation of blood by rolling it over the glass surface of an open vial in such manner that it does not churn in air bubbles and does not concentrate the hemoglobin appreciably by moisture evaporation. Have the apparatus set to receive the subsample. Pour a little more than 4 c.c. borax solution into the funnel cup. *Insure the absence* of minute air bubbles by drawing some of the solution into the reaction chamber and by gently shaking, turning the stopper and by forcing the mercury up and down. Bring the mercury column just up to the top of the upper stopcock bore, adjust the volume of the borax solution to exactly 4 c.c., and then let about 0.1 or 0.2 c.c. come under the upper stopcock. Close the lower stopcock but leave the upper one open. Draw the blood up into a pipette having two graduation marks, the so-called Ostwald-Van Slyke pipette. When oxygen content is determined (in which case the sample is kept under oil), keep the tip of the pipette away from the oil-water interphase as well as from the bottom and wipe the oil from the pipette. Lower the pipette down into the borax solution so that the tip comes a little above the stopcock. Let the blood run slowly into the borax solution. The blood will trickle through the bores and settle on the surface of the mercury. If the blood comes up over the tip of the pipette, it may follow the pipette when the pipette is withdrawn. Do not let the blood back up unnecessarily; an occasional quick turn of the lower stopcock is helpful in avoiding this. Introducing the blood sample does require care, but is not at all troublesome; good technicians do it neatly and quantitatively on the first trial. Immediately after the blood has been measured into the borax solution, cover it with 0.1 c.c. ferrous reagent. For this purpose also use a pipette with two graduation marks. Draw the reagent into the pipette from a few millimeters below the oil-water interphase, wipe off the oil, and then let the reagent flow out at a moderate rate just over the blood. Blood sample and ferrous reagent must not come into contact with the atmospheric oxygen. As the $FeSO_4$ enters the borax solution, $Fe(OH)_2$ precipitates as a very fine blue-green cast suspension. When the pipette is withdrawn, some of the flakes may be carried up by the current. Since there will be ample $Fe(OH)_2$ to meet

all the active oxygen present, these may be discarded. Those reaching the top will become contaminated and contribute to the ferric iron if they again settle; therefore, flush the mass of $Fe(OH)_2$ suspension on the bottom of the cup into the chamber before the floating $Fe(OH)_2$ particles settle. Draw the $Fe(OH)_2$ down into the reaction chamber with the borax solution so that 1.5 c.c. borax solution is measured in as accurately as can be done by the cup's graduation marks. Close both stopcocks, draw off the excess borate solution, and mix by gentle shaking. The reaction is complete within the first minute, but we let it stand for two minutes.

Any available oxygen in the reaction chamber will convert its equivalent mass of $Fe(OH)_2$ to $Fe(OH)_3$. Rinse the cup with three portions of water and *pipette** 2 c.c. 30 per cent sulfosalicylic acid into the cup, raise the levelling bulb, and open first the upper stopcock and then the lower so as to admit the fluid into the acid *slowly*. As the reaction mixture flows into the cup, stir it constantly with a slender rod until it is all well up in the cup. Draw off the colored, turbid fluid with a pipette having a rubber bulb and transfer it to a 15 c.c. graduated conical centrifuge tube. Rinse the cup with small portions of water until the meniscus of the fluid accumulating in the conical tube is just at the 10 c.c. mark, add a pinch of celite (filter aid), shake well, let stand a few moments for the precipitate to organize, and then filter through iron-free paper. Read the *perfectly clear*† iron-sulfosalicylate color density in a calibrated scopometer, and on its calibration graph note the volume per cent oxygen corresponding to the shutter scale reading.

CALCULATION

One molecule oxygen oxidizes 4 atoms ferrous iron; 1 mg. iron, therefore, is equivalent to 0.144 mg. O_2, the mass of oxygen occupying 0.1 c.c. at 0° C. and 760 mm. Hg. The blood oxygen would seldom reach 30 vol. per cent, and that may be taken as a high level for standards; this would be represented by 0.3 mg. Fe per cubic centimeter. If 259.2 mg. ferric ammonium sulfate crystals ($FeNH_4(SO_4)_2 \cdot 12H_2O$) are dissolved in water, made acid with 4 c.c. 30 per cent sulfosalicylic acid, and diluted to just 100 c.c., it will form a stock solution of such a concentration that when 1 c.c. of this is taken and 2 c.c. 30 per cent sulfosalicylic acid and 7 c.c. water are added, it will give a 10 c.c. colored solution with a tint and color density which are identical to a filtrate from a blood sample containing 30 vol. per cent available oxygen. This stock standard properly diluted to give lower oxygen levels was used to calibrate the instrument and may be used to make comparison solutions for visual colorimetry by the Duboscq type instrument. These standards are easily made up and keep very well.

Since the reagents are all aerated at atmospheric pressure and room temperature and the apparatus is rinsed with aerated water, there will be molecular oxygen to oxidize Fe++ besides that from the blood sample. A "blank" must, therefore, be done to account for this oxygen and such other consistent errors as may be involved. From the literature and from our solubility determinations

*The salicylic acid is measured with a pipette since marked differences in acidity affect the color density of iron salicylate solutions. There must not be any air bubbles introduced with the acid.

†Refilter the colored solution if a turbidity is visible in it.

of oxygen dissolved in water, the blanks in the regular procedures as herein described should be close to 9.8 volume per cent, but they usually fall between 8.6 and 9.3. To establish the blank, and this should be done frequently, proceed exactly as in the blood oxygen determination described, only omitting the 0.1 c.c. blood. Find the equivalent blood oxygen volume per cent and subtract this blank from that found in the regular routine procedure. When the oxygen is determined on aerated blood, to give the oxygen capacity or the normal active hemoglobin, the sample will carrry an extra 0.5 vol. per cent which is added to the blank before the subtraction.

RESULTS

It has been assumed that the Van Slyke-Neill manometric method[6] gives the true available oxygen content. The Van Slyke-Neill method gives as true values for oxygen as can be obtained in blood at the present time. As far as precision is concerned, one is not justified in seeking another procedure, but the manometric method calls for special skill, requires more blood than is sometimes convenient or available, and the apparatus is prone to unaccountable time-wasting leaks. The Van Slyke-Neill manometric blood oxygen method is a general approved means of standardizing hemoglobin determination methods; by the same token the oxygen capacity method for blood may be standardized by a hemoglobin determination *if* the hemoglobin is assured as being all normal and saturated with oxygen at proper temperature and pressure. For these reasons we have chosen to compare our new method with that published by Van Slyke and his co-workers and also with the hemoglobin in the samples as determined in our laboratory.

The acid hematin method[7] for determining hemoglobin has been very satisfactorily applied in our laboratory for more than twenty years. This is done by merely diluting 0.1 c.c. blood with 20 c.c. 0.1 *N* HCl and reading the color density twenty minutes after the dilution in a calibrated scopometer. Some modification of this way of determining hemoglobin is followed in most clinical laboratories. Time curves show that the maximum acid hematin concentration does not occur before the end of twenty minutes; the change between twenty and sixty minutes at room temperature is very little, if any.* The instrument is calibrated and the calibration occasionally checked with blood of known iron content. Because of the simplicity and the small samples required, there were more comparisons of the new oxygen method with this hemoglobin method than with the Van Slyke-Neill procedure in the first months of the program. This is justified by the close agreement between the Van Slyke-Neill method and the acid hematin values. The average deviation from the mean of duplicates by the former is 0.177 and by acid hematin (Hb. $\times$ 1.34) it is 0.166 vol. per cent, and the agreement between the two methods is expressed by $\pm$0.3 vol. per cent, which is derived from four groups of analyses in which the numbers of samples were 127, 38, 123, and 157 and their average deviations from their means, 0.36, 0.23, 0.312 and 0.275.

Where there was a lack of agreement, the Van Slyke-Neill method more often checked the acid hematin than did our new procedure, but this has not

*Increase in 0_2 from twenty to eighty minutes averages 0.16 vol. per cent; the average deviation from the mean at twenty minutes is 0.04, and at eighty minutes, 0.07.

been an invariable rule as the selected samples in Table I indicate. Since each method has a lesser deviation among duplicates than between any two of the three procedures, the differences by methods must be due to some inherent quality of the blood sample if bad checks in duplicates do not disqualify the data as due to manipulative error. The first four are typical of discordance; the next two are caused by a known inherent quality in the sample (namely, methemoglobin).

TABLE I

ACID HEMATIN (VOL. %)	COLORIMETRIC (VOL. %)	MANOMETRIC (VOL. %)	ACID HEMATIN (VOL. %)	COLORIMETRIC (VOL. %)	MANOMETRIC (VOL. %)	ACID HEMATIN (VOL. %)	COLORIMETRIC (VOL. %)	MANOMETRIC (VOL. %)
20.1	19.2	19.7	29.5	27.2	27.2	18.5	20.3	20.0
18.5	17.5	18.7	21.3	20.5	20.8	20.2	20.3	21.0
19.6	18.4	19.5	19.4	20.0	19.9	17.2	17.3	17.9
23.4	22.7	23.5	19.1	19.8	19.8	17.6	17.7	18.6

The acid hematin gives all the hemoglobin, and there may be some of this present which does not carry oxygen (methemoglobin). This discrepancy is a measure of the inactive hemoglobin. Blood samples should be examined promptly for available oxygen since the hemoglobin may change on standing. Methemoglobin in excess of 0.5 vol. per cent is not often encountered in random fresh blood samples,[8] but if these samples are set aside for some days, methemoglobin may form. The following samples were in good agreement between the acid hematin and direct oxygen determinations on the freshly drawn samples when they were put away in the cold chest, but on standing the oxygen values decreased to yield the following in these three sets: 15.2 to 14.0 vol. per cent; 15.6 to 14.8; 15.6 to 12.5. Sometimes the available oxygen remains constant or increases slightly as water evaporates, as in the following: Fresh blood sample, by the new method, May 14, 15.8 vol. per cent; May 17, 15.7; May 18, 15.9; May 19, 16.3; May 21, 16.5; May 24, 16.6. The acid hematin determination on the fresh blood sample gave a value of 15.6 vol. per cent. Three blood samples have also been encountered in which the oxygen content decreased on standing but was fully recovered again on aeration, a spontaneous deoxygenation. Methemoglobin does not vitiate the oxygen determination by the Van Slyke method nor by the new procedure, so that both methods may also be used in estimating the portion of hemoglobin which has been deprived of its oxygen transport capacity.[9] A methemoglobin blood preparation was made by treating a normal blood with ferricyanide. One cubic centimeter of this preparation was then added to 2 c.c. of an aerated normal blood sample and the oxygen determined. The oxygen in this sample by the new method before adding the methemoglobin preparation came to 19.6 vol. per cent and to 19.2 by the Van Slyke-Neill method; that calculated from acid hematin hemoglobin was 19.6. Two-thirds of their average would be 13.0. By the actual determinations after mixing, they came to 13.6, 13.6, and 17.9, using the same methods and following the same order. In this set the acid hematin value minus the direct oxygen determinations comes to 4.3 vol. per cent; this value divided by 1.34 gives 3.2 Gm. methemoglobin per 100 c.c. of the mixture.

Oxygen was determined on many kinds of blood from the very beginning of this general problem, but until the method was believed to be in its final form

and reliable, most of the samples examined were fully aerated. These data on aerated blood are summarized in Table II. The time periods are arranged in chronological order. The first period includes many of the experiments done during the development of the method itself. Subsequent to this period there have been no essential changes in chemistry nor manipulation. The second period is noteworthy in that it was a flawless run of both colorimetric and manometric determinations on fresh bloods with remarkable agreement between the two procedures.*

TABLE II

COMPARING THE NEW COLORIMETRIC METHOD FOR OXYGEN IN BLOOD WITH THE VAN SLYKE-NEILL AND/OR THE ACID HEMATIN METHODS ON AERATED BLOOD

PERIOD	NUMBER OF SAMPLES	MEAN OF DIFFERENCES BETWEEN TWO METHODS	AVERAGE DEVIATION FROM THE MEAN DIFFERENCE		PER CENT OF SAMPLES IN WHICH THE AGREEMENT CAME WITHIN	
			FOR ALL SAMPLES	FOR THOSE WITHIN ±1.0	±0.6	±1.0
I	416	+0.7	0.61	0.48	69	87
II	25	+0.036	0.10	0.10	100	100
III	220	+0.01	0.37	0.33	87	96

The subsequent routine determinations on aerated blood indicate that the new procedure gives results which agree with manometric determinations to within 0.5 vol. per cent in more than 85 per cent of the cases.

The application of the method to venous blood is summarized in more detail in Table III. These samples were obtained from volunteers among friends of the department in the Home Office, and we are duly grateful to them for the interest shown in our work. In most instances they came directly from their desks; a few were having metabolism tests and the samples were taken under basal control. They were all presumably normal subjects. The two tests were done simultaneously, the Van Slyke-Neill manometric method by Miss Weakley and the new colorimetric by Mr. Huizer. In this series we were also concerned with the oxygen exchange between the hemoglobin and the oil used to protect the blood from the oxygen in the atmosphere. The sample was protected from the atmosphere but not from the oxygen dissolved in the oil. Kubie[10] accepts the protective function of oil and explains it as due to the very slow diffusion of gas through the oil, not to exclusion from lack of solubility. He bubbled oxygen from a cylinder into mineral oil for a long period and in that way determined the capacity of the oil as 0.134 c.c. oxygen per cubic centimeter. We have determined the oxygen content of our oil supply on two days, four months apart, by both methods. The average for the manometric method came to 2.40 and 2.42 vol. per cent and by the colorimetric, 2.3 and 2.8, making an average of 2.47 vol. per cent for all the determinations. Fully aerated blood passing through this approximately fifth oxygen-saturated oil did not lose any oxygen, but samples containing any deoxygenated hemoglobin picked up oxygen. The oxygen in the samples reported in Table III were determined at once by the two

*The determinations in this series by the new method were done by Dr. F. Schattner and Mr. J. Huizer, and those by the Van Slyke-Neill methods, by Miss Mary C. McCarthy and Miss Mary L. Weakley. Grateful acknowledgment must also be made for the splendid and kindly cooperation of Dr. William Antopol of the Laboratory of the Newark Beth Israel Hospital, for supplying many blood samples.

TABLE III

	IMMEDIATE			THROUGH OIL BY DROPS			GAIN FROM OIL		OXYGEN SATURATION			AERATED BLOOD				L/M	L/N	L/O	N/M
	A (VOL. %)	B	C (%)	D (VOL. %)	E	F (%)	G (VOL. %)	H	I (VOL. %)	J (%)	K (%)	L	M (VOL. %)	N	O	P (%)	Q (%)	R (%)	S (%)
H. S.	6.9	6.74	102.4	8.9	8.77	101.0	1.0	2.0	15.8	40	39	17.3	17.92	17.2	–	96.6	100.5	–	96.2
H. S.	6.5	6.19	105.0	7.9	7.40	106.5	1.4	1.2	16.5	39	38	17.4	18.10	17.6	18.0	96.2	99.0	96.6	97.2
H. S.	8.8	9.17	96.2	10.0	9.97	100.3	1.2	0.8	15.9	55	58	16.6	16.60	17.0	16.8	100.0	97.8	99.0	102.3
J. C.	6.0	5.73	104.6	7.5	7.57	99.3	1.5	1.9	18.2	33	31	20.8	*22.22*	20.5	–	*94.1*	101.5	–	*92.3*
M. G.	5.2	4.60	113.0	6.8	6.40	106.2	1.6	1.8	16.2	32	28	17.7	18.11	17.6	–	98.0	100.5	–	97.2
T. L.	6.5	6.47	100.5	8.1	7.96	101.5	1.6	1.5	17.1	38	38	17.7	*18.69*	17.6		*94.8*	100.5	–	*94.2*
M. A.	4.9	4.49	109.0	6.1	5.59	109.0	1.2	1.1	15.3	32	29	15.8	16.23	16.3	–	97.5	97.0	–	100.4
M. A.	6.1	5.64	108.0	–	–	–			15.3	40	37	15.6	15.30	15.3	15.3	102.0	99.0	102.0	100.0
H. M.	8.7	8.98	97.0	10.1	10.28	98.2	1.4	1.3	20.9	41	43	21.9	21.77	21.5	–	100.5	101.8	–	98.8
F. K.	9.5	9.97	97.4	10.7	10.78	99.2	1.2	1.0	20.8	46	47	22.3	*23.03*	*20.0*	22.0	*97.2*	*107.2*	101.3	*90.5*
M. K.	6.3	9.54	113.6	7.5	6.90	108.6	1.2	1.4	18.0	35	30	19.8	19.80	*18.0*	19.1	100.0	*110.0*	103.5	*90.6*
J. B.	12.3	12.99	94.8	13.7	13.74	100.0	1.4	0.7	19.4	63	67	20.9	20.80	21.2	20.6	100.5	98.6	101.3	101.9
P. P.	9.3	8.90	104.3	9.8	10.16	96.2	0.5	1.2	18.8	50	47	19.5	19.65	*18.8*	19.6	99.2	*103.7*	99.5	*95.8*
P. P.	7.9	7.30	108.0	9.1	8.55	105.5	1.2	1.4	20.0	39	37	21.5	20.96	*20.0*	21.0	102.5	107.4	102.4	*95.8*
J. C.	12.0	12.27	97.6	13.4	13.57	98.6	1.4	1.2	18.7	62	66	19.6	*20.70*	19.8	20.4	*95.0*	99.0	96.2	95.7
E. B.	9.0	9.10	99.0	9.8	9.97	98.6	0.9	0.8	19.6	46	51	20.5	21.20	20.7	21.5	96.8	99.0	95.2	97.7
R. W.	*13.4*	13.90	*96.4*	15.4	15.0	102.5	2.1	1.1	19.3	69	72	20.4	21.10	20.5	20.8	96.8	99.8	98.0	97.2
M. R.	7.1	7.00	101.5	7.8	7.94	98.4	0.7	0.9	19.5	36	39	20.5	20.97	20.4	20.6	98.0	100.2	99.8	97.4
A. R.	6.5	6.49	100.2	7.6	7.69	99.0	1.1	1.2	17.1	38	38	18.1	18.37	17.8	17.4	98.6	101.7	104.0	97.2
A. R.	3.9	3.98	98.0	5.8	*5.09*	*113.5*	1.9	1.1	*16.5*	24	24	16.7	16.85	*17.5*	16.6	99.0	*95.5*	100.5	*103.6*
W. F.	2.9	2.68	108.0	4.3	3.84	111.8	1.4	1.2	19.5	15	14	20.9	20.50	20.9	20.8	102.0	100.0	100.5	102.0
D. C.	10.8	11.60	93.0	12.5	12.5	100.0	1.7	0.9	20.5	52	55	22.7	22.50	*21.9*	22.4	101.0	*103.5*	101.2	97.3
S. C.	13.8	14.05	98.8	14.3	14.4	99.5	0.4	0.4	18.8	74	74	20.4	21.05	20.5	20.9	97.2	99.8	97.7	97.4
N. S.	2.7	2.69	100.5	3.7	3.36	110.0	1.0	0.7	16.5	16	16	*16.8*	17.57	17.2	17.6	*95.5*	*97.8*	*95.5*	97.8
S. S.	12.8	13.30	96.5	14.0	14.5	96.5	1.2	1.2	18.0	71	74	19.1	19.70	19.3	*20.6*	97.0	99.0	*92.8*	98.2
W. S.	8.8	8.78	100.0	9.7	9.67	100.0	0.9	0.9	19.4	45	45	20.0	20.07	20.4	20.0	100.0	98.0	100.0	102.0
A. W.	9.8	10.29	95.0	10.7	*9.67*	*110.8*	0.9	*-0.5*	18.5	53	56	18.5	18.50	19.2	19.0	100.0	96.4	97.5	103.7
J. H.	10.4	10.67	97.5	11.8	11.58	102.0	1.4	0.9	18.8	57	57	19.4	19.70	19.4	19.6	98.6	100.0	99.0	98.5
J. H.	7.3	7.50	97.5	8.7	9.06	96.0	1.4	1.6	19.3	38	39	–	–	–	–	–	–	–	–
J. H.	11.0	11.06	99.6	12.2	12.30	99.2	1.2	1.1	17.3	64	64	17.3	17.40	17.8	17.2	99.6	97.2	100.5	102.0
J. H.	12.2	11.88	102.5	13.2	13.36	98.8	6.8	1.5	17.6	70	68	18.5	19.20	19.0	19.2	96.5	97.2	96.5	99.0
Average			100.4			102.3										98.4	100.2	99.5	101.4
			100.2			101.9										98.7	99.5	99.5	99.7
Difference Mean	0.040			0.050								0.150							
Average deviation	0.307			0.236								0.335							

methods. The "immediate" oxygen values are given under *A* and *B*. When the "immediate" oxygen had been determined, a syringe tip was put into the vial, some oil was drawn in and then the blood. The blood was then slowly pressed out in as small drops as possible under oil into another vial. This was repeated. Every caution was taken to keep the sample from direct contact with the atmosphere. Two subsamples were then taken and the "by drops through oil" oxygen determined and recorded under *D* and *E*. The increment between these two sets is an index of the contamination from the oil. The initial oxygen contribution to the blood sample from the oil, even also that from the empty needle as the blood is drawn from the vein, is probably no more than this increment; nevertheless it is real, averaging 1.21 mg. vol. per cent for the colorimetric column (*G*) and 1.18 for the manometric column (*H*) in Table III, with an average deviation of 0.28 from the mean for both columns, a possible contamination of the order of 10 per cent.

The main purpose of the venous blood analysis gathered in Table III was again to compare the new method with that of Van Slyke and Neill on the kinds of blood samples encountered in the clinical laboratory and used in physiologic investigations. The colorimetric data are listed under *A*, *D* and *L;* the manometric, under *B*, *E* and *M*. The hemoglobin was determined by acid hematin and read directly as equivalent oxygen volume per cent on a calibration chart; the hemoglobin in grams was multiplied by 1.34. The data for the blood as drawn are listed under *I* and those after aeration, under *N*. The iron[11] was also determined on the aerated blood and the milligrams per 100 c.c. multiplied by 400 to give equivalent volume per cent oxygen; these are listed in column *O*.

From *I* and *A* and *B* the oxygen saturation of the venous hemoglobin is calculated and listed under *J* for the new method and under *K* for the Van Slyke-Neill figures. These saturations cover a rather wide range, from 14 per cent for W. F. to 74 per cent for S. C., much wider and lower than those reported by Lundsgaard and Möller,[12] whose lowest saturation was 48 per cent and highest 96 per cent with a median at 77 per cent instead of 41 as in Table III; 78 per cent of their cases fall between 60 per cent and 90 per cent. In our list one-half of the cases fall between 38 and 58 per cent saturation. Any further comments on this matter will be left for the time being and taken up again when the data now accumulating in this area are published.

The determined volume per cent by the new colorimetric method (*A*, *D* and *L*) were multiplied by 100 and then divided by the values obtained with the Van Slyke apparatus (*B*, *E* and *M*) to give the percental ratios in columns *C*, *F*, and *P*. The small divergence of these *per cents* from 100, averaging *C*, 0.4; *F*, 2.3; and *P*, 1.6; prove the two methods to be in tolerable agreement. If one goes over Table III critically, one may cull values that may be discredited (those in italics) as, for instance, the case of N. S. with *M*, *N*, *O* values within a spread of 0.4 vol. per cent and the *L* value 0.6 below their average. In this instance the value by the new method is conceded as most likely too low; in four other subjects, on the other hand, the manometric figures are thought rather high if credence be not denied the iron and acid hematin readings (J. C., T. L., F. K.). Neither the acid hematin nor iron method is infallible. In the samples

from F. K. and M. K. the acid hematin figures (*N*) are the same as those before aeration (*I*), which is not in accord with experience. If due allowances are made for this way and the averages are recalculated with the questioned cases omitted, these figures come closer to 100 and are entered at the bottom of Table III. The improvement by thus selecting the cases is not significant. When the new method is compared with the acid hematin and the iron procedures in the same manner, we get columns *Q* and *R* by dividing $L \times 100$ by *N* and *O*. These averages come to 100.2 and 99.2 and are again in excellent agreement. Aeration of the samples seemed to enhance the oxygen values by the manometric method, for which reason the percentile ratio of acid hematin/manometric methods were calculated and listed in column *S*. The average of *S* agrees well with that of *P*. A specific effect of aeration on one of the oxygen determinations may be real but is quantitatively insignificant for the purpose and therefore dismissed for the present. The two methods were also compared by their differences in volume per cent, *A-B*, *D-E*, and *L-M*. The average deviations from the mean differences are given at the bottom of Table III. The eight italicized oxygen values are omitted in these averages. The range of those used are from −0.80 to +0.76. Two samples among those omitted would extend the range from −1.40 to +1.00. Less than 15 per cent of the cases exceed a difference of 0.5 vol. per cent, and the average difference of all eighty-three pairs in this table is less than 0.3 vol. per cent.

SUMMARY

A colorimetric method for the determination of available oxygen in 0.1 c.c. sample of blood from a vein or finger prick is presented. The method depends on the oxidation of ferrous iron by the oxygen of the blood sample to ferric iron and then combining this ferric ion with sulfosalicylic acid to yield a characteristic and consistent color whose density under the conditions of the test bear a direct relation to the amount of oxygen in the sample. The oxidation of iron takes place in a solution made alkaline with borax in an apparatus designed to protect it from atmospheric oxygen. The sulfosalicylic acid prevents further oxidation and removes the proteins. The color density may be compared with known ferric sulfosalicylate solutions in a visual instrument of the Duboscq type or may be measured in a calibrated photoelectric instrument like the scopometer. Oxygen values by this method have been compared with simultaneous determinations with the Van Slyke-Neill manometric method and with oxygen equivalents calculated from hemoglobin and iron determinations. The agreements of the several methods are of the order of ±0.5 vol. per cent in 85 per cent of the cases.

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BOOK REVIEWS

Atlas of the Blood in Children. By *Kenneth D. Blackfan,* M.D., Late Thomas Morgan Rotch Professor of Pediatrics, Harvard Medical School; Late Physician-in-Chief, Infants' and Children's Hospitals, Boston; and *Louis K. Diamond,* M.D., Assistant Professor of Pediatrics, Harvard Medical School; Visiting Physician and Hematologist, Infants' and Children's Hospitals, Boston; with illustrations by *C. Merrill Leister,* M.D., Associate Pediatrician, St. Luke's Hospital, Bethlehem, Pa., and Allentown General Hospital, Allentown, Pa. The Commonwealth Fund, New York, N. Y. Price $12.00. Cloth with 334 pages and 70 plates.

The seventy color plates about which this atlas is built are unquestionably the finest reproductions of peripheral blood films stained with Wright's stain that have so far been printed as a collection in this country. They are at least the equal of the excellent color plates which appeared in Downey's *Handbook of Hematology.* The illustrations were painted by Dr. C. Merrill Leister; 560 separate plates, each reproduced in eight colors, were made for the seventy full-page plates. Reproductions of color tone, of nuclear structure, and of cytoplasmic inclusion bodies are amazingly accurate. Eosinophilic and neutrophilic granules in several instances appear slightly diagrammatic, but this is a minor objection. A student or a physician trying to identify blood cells on a slide in which young or abnormal forms of cells are present would receive greater help from this atlas in his attempt to identify the cells which puzzle him than from any other source now available.

In addition to the color illustrations, there are a number of charts, 144 pages of text, and a comparatively short bibliography. The text itself, written by Drs. Blackfan and Diamond, is characterized by a simplicity and clarity of expression which is unusual in hematologic writings. Following a brief description of the blood in infancy and childhood, the authors discuss the etiology, the clinical manifestations, and diagnosis and treatment of the anemias, the diseases involving white blood cells, and the purpuras. For each dyscrasia, one or two cases histories, carefully selected to illustrate problems of diagnosis and treatment, are given. While the book concerns itself primarily with hematologic abnormalities in children, much of the information will prove valuable to the internist as well as to the pediatrician and student. It should be pointed out, however, that it does not purport to be a complete text of blood disorders. The lymphomas other than lymphatic leucemia and the hemorrhagic diseases other than purpura are discussed very briefly since they do not cause characteristic cytologic changes in the peripheral blood. Discussion is limited entirely to conditions with which the authors have had close personal experience.

While the reviewer does not wish to detract in any way from the impression he hopes to give of the book's fine qualities, he should record a few omissions

which seem unfortunate. Nothing is said in the section devoted to the treatment of congenital hemolytic anemia of the possible danger of accelerating hemolysis by giving transfusions prior to splenectomy. Sicklemia, or the sickle-cell trait, is not sharply differentiated from sickle-cell anemia. Most readers would appreciate knowing the percentage incidence of the various types of leucemia seen by the authors in their wide experience. The appearance of the megakaryocytes in the bone marrow of children with primary thrombocytopenic purpura is not described; many hematologists regard this cytologic change as moderately specific. Auer bodies are neither mentioned nor illustrated.

The book is beautifully printed on fine paper. Anyone acquainted with the cost of color illustrations, particularly those printed in eight colors, will recognize how moderate is the price of $12. As a matter of fact, every reader should be aware of his debt not only to the authors, but also to the Commonwealth Fund for having shared so large a percentage of the expense. The atlas probably represents the last major publication of work in which the late Dr. Blackfan participated. It could be considered a worthy memorial.

Secretory Mechanism of the Digestive Glands. By *B. P. Babkin*, M.D., D.Sc., LL.D., F.R.S.C., Research Professor of Physiology, McGill University, Montreal, Can.; Formerly Professor of Physiology in the University of Odessa, Russia, and in Dalhousie University, Halifax, Nova Scotia, Can. With 220 illustrations. Paul B. Hoeber, Inc., New York, N. Y. Price $12.75. Cloth with 900 pages.

Dr. Babkin has brought out a book on the secretory mechanism of the digestive glands based upon several courses of lectures given by him during the last ten years to postgraduate students at McGill University and some lecture-reviews presented at University College, London, and at the Universities of Edinburgh and Birmingham. Much of the material for this book is derived from work performed by him and his co-workers. There is a great deal of discussion of the secretion of the gastric juice and of the gastric mucus. In addition there is a detailed discussion of the secretion of the saliva, a subject which has not often been treated in such detail in many texts.

The book is well illustrated and contains a hugh bibliography. It is an excellent review for those men interested in the mechanism of the secretion of the digestive glands and will be of particular value to research laboratories. In view of the marked revival of interest in both theoretical and clinical gastroenterology during the last few years, it should prove useful to the gastroenterologist doing research and teaching, but the book will have little value to the average clinician.

C. J. B.

An outline of Tropical Medicine. By *Otto Saphir*, M.D., Director of the Department of Pathology of the Michael Reese Hospital; Professor of Pathology, University of Illinois College of Medicine. Michael Reese Foundation, Chicago, Ill. Cloth with 86 pages.

An Introduction to Public Health. By *Harry Stoll Mustard,* Director DeLamar Institute of Public Health, College of Physicians and Surgeons, Columbia University. Second edition. The Macmillan Co., New York, N. Y. Price $3.25. Cloth with 283 pages.

Internes' Handbook. By *M. S. Dooley,* M.D., A.B., Professor of Pharmacology, and *Maynard E. Holmes,* M.D., F.A.C.P., Professor of Clinical Medicine, Third edition. J. B. Lippincott Company, Philadelphia, Pa. Price $3.00. University of Syracuse Medical School. Cloth with 579 pages.